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Morphogenesis and evolution of annuli in arthropod appendages

Morfogenesi ed evoluzione degli annuli nelle appendici degli artropodi

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RIASSUNTO

Gli articoli che compongono le appendici degli artropodi vengono generalmente distinti in due tipologie contrapposte: *articoli veri* ed *annuli*. Gli annuli sono generalmente definiti come articoli privi di muscolatura intrinseca, cioè senza alcuna inserzione muscolare al loro interno. Inoltre, gli annuli sono spesso considerati “suddivisioni secondarie” di articoli veri. Oltre a queste differenze anatomiche, è stata recentemente anche notata una generale differenza nell’ontogenesi tra annuli ed articoli veri, notando che gli annuli tendono a formarsi relativamente più tardi nello sviluppo. Anche specifici studi di biologia dello sviluppo sulle appendici di *Drosophila* sembrano supportare una differenza tra articoli veri ed annuli.

Il presente progetto mira ad indagare alcuni aspetti dell’ontogenesi degli annuli per capire: 1) quali siano, se presenti, le somiglianze nello sviluppo degli annuli di appendici diverse, entro la stessa specie e tra specie diverse di artropodi e 2) quali siano, se presenti, le differenze nello sviluppo tra annuli ed articoli veri. A questo scopo ho scelto di concentrare la ricerca su due aspetti principali: a) il rapporto tra muscoli, inserzioni muscolari ed articolazioni e b) il processo di annulazione nelle strutture flagellari (parti terminali di un’appendice composte unicamente da annuli) ed il suo rapporto con la crescita a livello cellulare. Il rapporto tra inserzione dei muscoli ed articoli è cruciale nella definizione di annuli fornita sopra. Per gli articoli veri più studiati, quelli delle zampe degli insetti, ci sono indizi che mostrano un legame di sviluppo piuttosto stretto tra il differenziamento delle cellule della membrana artrodiale (cellule epidermiche che producono l’articolazione) e quello delle inserzioni muscolari. Ad ogni modo, un certo livello di variabilità e di indipendenza tra le articolazioni e le inserzioni muscolari è atteso, dato che gli annuli, per definizione, sono articolazioni senza alcuna inserzione muscolare. Parti di appendici composte da annuli mostrano spesso un aumento nel numero di questi fin tanto che ci sono mute nella vita dell’animale. Il meccanismo mediante il quale avviene questo aumento è stato chiarito solo per poche specie ed esclusivamente per le antenne. Una stretta correlazione tra crescita di queste parti e loro segmentazione (termine qui usato nel senso di formazione di nuovi articoli) è inoltre stata notata nei pochi casi in cui c’è stato uno studio specifico, ma poco è noto per ciò che riguarda lo sviluppo dell’epidermide (divisione cellulare, differenziamento ed apoptosi) durante la segmentazione.

Per studiare il rapporto tra inserzioni muscolari ed articolazioni sono stati scelti diversi modelli: le appendici naupliari (antenne prime ed il solo esopodite delle antenne seconde e delle mandibole) del crostaceo cirripede *Balanus improvisus* Darwin, 1854, l’esopodite delle antenne seconde dei nauplii del crostaceo branchiopode *Artemia* sp., le antenne del centopiedi *Lithobius forficatus* (Linnaeus, 1758) ed i rami dei pleopodi del crostaceo malacostraco *Gammarus roeselii* Gervais, 1835. Per questi modelli l’articolazione, il sistema muscolare ed i relativi cambiamenti durante lo sviluppo postembrionale sono stati considerati. Inoltre, è stata riveduta in dettaglio la letteratura riguardante le appendici naupliari di tutti i maggiori gruppi di crostacei.

Sia le antenne prime che l’esopodite delle antenne seconde e delle mandibole dei nauplii di *B. improvisus* hanno muscoli che decorrono paralleli all’asse prossimo-distale per tutta la lunghezza dell’appendice; ciascuno di questi

muscoli ha inserzioni corrispondenti ad ogni confine articolare. L'esopodite delle antenne seconde e delle mandibole aumenta il numero di articoli durante lo sviluppo naupliare e la formazione dei nuovi confini articolari è accoppiata con la formazione di inserzioni intermedie lungo l'asse di muscoli già esistenti. Condizioni molto simili si ritrovano, di regola, anche nelle appendici naupliari di altri crostacei.

Risultati inattesi sono stati ottenuti dallo studio anatomico dell'esopodite delle antenne seconde dei nauplii di *Artemia*. L'esopodite ha, sul lato postero-ventrale, 8-10 sete natatorie (numero con variazione individuale) che, alla base, producono pieghe cuticolari molto simili a quelle articolari; sul lato opposto ci sono pieghe cuticolari il cui numero varia a livello individuale, da 8 a 14. Numero e posizione delle sete e delle pieghe cuticolari del lato opposto non corrispondono e quindi, mancando un limite continuo ed uniforme, non si possono individuare veri e propri articoli nell'esopodite. Tre muscoli decorrono lungo l'intero esopodite, parallelamente all'asse prossimo-distale; questi muscoli hanno inserzioni alla base di una seta (per i due muscoli che decorrono da quel lato) o su una piega cuticolare (per il singolo muscolo che decorre dal lato opposto) e dato che le pieghe cuticolari alla base delle sete non corrispondono alle pieghe cuticolari del lato opposto, questa non corrispondenza si riflette anche nelle inserzioni muscolari.

Sia le antenne di *L. forficatus* che i rami dei pleopodi di *G. roeselii* hanno muscoli (rispettivamente quattro e due) che decorrono parallelamente all'asse prossimo-distale lungo tutta la loro lunghezza; questi muscoli hanno inserzioni intermedie in corrispondenza di ogni confine articolare.

Nonostante gli articoli delle antenne di *Lithobius* siano generalmente considerati articoli veri, mentre quelli dell'esopodite delle antenne seconde e delle mandibole dei nauplii di *Balanus* (ed altri crostacei) e dei rami dei pleopodi di *Gammarus* siano considerati annuli, non si notano differenze nella presenza o assenza di inserzioni muscolari; le differenze anatomiche presenti riguardano piuttosto la struttura delle inserzioni muscolari (quantità di matrice tendinea) e dei confini articolari (estensione della membrana artrodiale). Tutte le strutture che aumentano il numero di articoli durante lo sviluppo postembrionale da me analizzate o discusse, producono nuovi confini articolari accompagnati, di regola, da nuove inserzioni intermedie di muscoli già esistenti.

Il meccanismo di formazione di nuovi annuli è stato studiato con un certo dettaglio per il flagello delle antenne seconde dei crostacei isopodi. *Asellus aquaticus* (Linnaeus, 1758), principale modello, è stato studiato in dettaglio sia durante il normale sviluppo postembrionale che durante la rigenerazione; altre specie studiate sono state *Idotea chelipes* (Pallas, 1766), *Lirceus fontinalis* Rafinesque-Schmaltz, 1820 e *Sphaeroma serratum* (Fabricius, 1787). La maggior parte del flagello di *A. aquaticus* (Asellidae) è composto da "quartetti": unità di quattro articoli, ciascuno dei quali con sete in numero e posizione caratteristici. Nuovi articoli e quartetti sono prodotti, durante l'intera vita dell'animale, nella parte prossimale del flagello: l'articolo più prossimale si divide producendo articoli che (entro certi limiti indipendentemente tra loro) si dividono tre ulteriori volte producendo così un quartetto. In *A. aquaticus* il meccanismo di formazione di nuovi annuli è stato studiato anche durante la rigenerazione ed è pressoché identico a quello dello sviluppo normale a prescindere dal livello in cui è avvenuta l'amputazione. In *L. fontinalis* (Asellidae) la maggior parte del flagello è composta da “duetti”: coppie di articoli, ciascuno dei quali porta sete corrispondenti a due articoli di un quartetto di *A. aquaticus*. Il meccanismo di produzione è anch’esso simile ma gli articoli prodotti dal più prossimale si dividono una volta soltanto. Una certa variabilità è però presente e sono ritrovabili anche “terzetti” (da articoli prodotti dal più prossimale che si sono divisi due volte) e veri e propri quartetti (da articoli prodotti dal più prossimale che si sono divisi tre volte) in tutto e per tutto identici a quelli di *A. aquaticus*. In *S. serratum* (Sphaeromatidae) la maggior parte del flagello presenta articoli subeguali. Il meccanismo di produzione di nuovi articoli prevede la divisione dell’articolo più prossimale ed un’ulteriore divisione dell’articolo prodotto da questo. In *I. chelipes* (Idoteidae) la maggior parte del flagello ha articoli subeguali e nuovi articoli sono prodotti unicamente dalla divisione dell’articolo più prossimale, contrariamente a *S. serratum*.

Il meccanismo di formazione di nuovi annuli ed il suo rapporto con la crescita a livello cellulare è stato studiato in due modelli: il flagello delle antenne seconde di *A. aquaticus* ed i rami dei pleopodi di *G. roeselii*. I rami dei pleopodi di *G. roeselii* aumentano il numero dei loro articoli per tutta la vita dell’animale. Nuovi articoli sono prodotti nella parte più prossimale del ramo, dalla divisione del primo articolo soltanto; in queste appendici, così come nel flagello delle antenne II di *A. aquaticus*, figure mitotiche sono state identificate solo nella parte più prossimale. Le parti più distali (e quindi “di età maggiore”) mostrano nuclei mediamente più allungati: c’è quindi una zona proliferativa prossimale da cui le cellule prodotte vengono continuamente “spinte” verso posizioni più distali dalla formazione di nuove cellule subendo un cambiamento di forma. Attualmente non è ancora compreso come questo processo sia coordinato con il meccanismo di produzione di nuovi articoli.

La produzione di articolazioni e di inserzioni muscolari sono processi di sviluppo correlati. Dati a supporto di ciò erano già disponibili per gli articoli delle zampe degli insetti (eccetto i tarsomeri) e nella presente tesi ho mostrato come questo sia vero anche per altre appendici di artropodi, dato che nuove articolazioni prodotte durante lo sviluppo postembrionale sono sempre accoppiate con nuove inserzioni muscolari, se muscoli sono presenti. Articoli senza alcuna inserzione muscolare possono essere presenti nelle appendici di artropodi, ma questi sono completamente privi di muscoli che li attraversano oppure sono attraversati unicamente da tendini. L’effettiva esistenza di articoli attraversati da muscoli ma privi di inserzioni è, al momento, molto dubbia. La tradizionale distinzione tra articoli veri ed annuli basata sulla presenza/assenza di inserzioni muscolari è errata: molti articoli tradizionalmente considerati annuli hanno, in realtà, inserzioni muscolari. Dal punto di vista funzionale, comunque, questa distinzione è tutt’ora valida dato che articoli con inserzioni intermedie di muscoli paralleli all’asse prossimo-distale non possono produrre movimenti reciprocamente indipendenti come, invece, possono fare gli altri articoli veri (con muscoli intrinseci antagonisti interamente confinati al loro interno). L’esopodite delle antenne seconde dei nauplii di *Artemia* fornisce ulteriori prove di uno stretto legame tra articolazioni ed inserzioni muscolari. In questo modello non sono presenti articolazioni complete, ma solo “parziali” pieghe cuticolari; queste, che derivano probabilmente da articolazioni complete, sono comunque provviste di inserzioni muscolari. Una generale differenza nell’ontogenesi tra annuli ed articoli veri era stata da altri autori, notando che gli annuli tendono a formarsi relativamente più tardi nello sviluppo. Nella presente tesi questo aspetto è stato ulteriormente discusso in dettaglio: questa differenza è reale ma non contrappone articoli veri ed annuli (se questi sono definiti in base alla presenza/assenza di inserzioni muscolari), bensì articoli con movimenti reciprocamente indipendenti ed articoli che agiscono all'unisono. Anche questa differenza ontogenetica, comunque, si può ricollegare alla differente morfologia funzionale di questi articoli. Strutture flagellari mostrano interessanti somiglianze nello sviluppo postembrionale; queste somiglianze sono spiegabili dalla comune presenza di una "zona di crescita" prossimale (una zona dove sono prodotti nuovi articoli e sono localizzate le mitosi dell'epidermide). La distribuzione filogenetica di questa zona di crescita è discussa e, nella presente tesi, viene proposto che la presenza di una zona di crescita sia una condizione primitiva nello sviluppo postembrionale delle (prime) antenne e dei rami delle appendici postantennulari per lo meno degli artropodi mandibolati (miriapodi, insetti e crostacei).

ABSTRACT

A widely used distinction among articles that usually composed an arthropod appendage is the one between *true articles* and *annuli*. This distinction is often claimed to be based on the anatomy of the muscular system, true articles have intrinsic musculature while annuli do not. Annuli are also usually considered a subdivision of a true article. Recently, it has also been noted that annuli tend to be produced later during development. Observations on development of *Drosophila* appendages also seem to support a basic difference between the process that produce true article and the one that produce annuli.

In the present project I studied selected aspects of article anatomy and development, in order to understand: a) which are (if present) the developmental similarities among annuli of different appendages and different arthropods, and b) which are (if present) the developmental differences between annuli and true articles. I decided to focalise the research on two topics: a) the relationships between muscles, muscle insertions and joints, and b) the mechanism of annulation in flagellar structures (terminal part of an appendage composed of only annuli) and its relationship with growth at the cellular level. According to the definitions of true articles and annuli given above, the anatomy of the muscular system is the most important aspect. For the most studied true articles, those of insect leg, there is evidence of a close developmental relationship between the development of the arthrodial membrane cells (epidermal cells that produce the joint) and muscle insertions. However some variation is expected as annuli are supposed to be joint without any muscle insertion. Parts of appendages composed of only annuli often show indeterminate postembryonic increasing in the number of annuli. The mechanism by which new annuli are produced has been studied only in few species or groups, and only for the antennae. Where both the mechanism of article production and the overall growth have been studied, a close relationship between the two was noted, but little is known about the development of the epidermis (cellular division, differentiation and apoptosis) during segmentation.

Different models have been employed to study the relationships between muscles, muscle insertions and joints and these are: the naupliar appendages (first antennae and exopod of both second antennae and mandibles) of the cirriped crustacean *Balanus improvisus* Darwin, 1854, the exopod of the naupliar second antennae of the branchiopod crustacean *Artemia* sp., the antennae of the centipede *Lithobius forficatus* (Linnaeus, 1758) and the rami of the pleopods of the malacostracan crustacean *Gammarus roeselii* Gervais, 1835. In these models the segmentation, the muscular system and the postembryonic changes have been studied. Literature on naupliar appendages anatomy and postembryonic development has also been reviewed in detail.

There are some muscles running parallel to the proximo-distal axis throughout the first antennae and the exopod of both second antennae and mandibles in the nauplii of *B. improvisus*. These muscles have insertions on every joint. The exopod of both second antennae and mandibles increase in article number during naupliar development and new joints have new intermediate insertion of already present muscles. Very similar conditions are usually found in the naupliar appendages of other crustaceans.

Unexpected results have been obtained on the exopod of naupliar second antennae of *Artemia*. The exopod has 8-10 natatory setae (number with individual variation) on the posterior-ventral side, which have some cuticular folds at their base, resembling a joint; on the opposite side there are 8-14 (number with individual variation) cuticular folds. Number and position of setae and cuticular folds do not match and thus complete joints are lacking. Three muscles are present within the exopod; they run parallel to the proximo-distal axis and have insertions at the base of a seta (for the two muscles that are on that side) or on a cuticular fold (for the single muscle that run on that side). Since setae and cuticular folds do not match, there is mismatch also in the muscular insertions of the two sides.

In the antennae of *L. forficatus* there are four muscles that run parallel to the proximo-distal axis throughout their length. These muscles have an insertion on each joint. The rami of the pleopods of *G. roeselii* have two muscles that run parallel to the proximo-distal axis throughout their length, with insertion on each joint.

Thus, even if the articles of the antennae of *Lithobius* are usually considered true articles and those of the naupliar exopod of second antennae and mandibles of *Balanus* (and other crustaceans) as well as those of the rami of the pleopods of *Gammarus* are usually considered annuli, there is no difference on the presence/absence of muscular insertions. Anatomical differences are present in the structure of the muscular insertion (tendon matrix) and of the joint (extent of arthrodial membrane). All the appendages originally studied here or those discussed in the review that increase in article number during postembryonic development produce new joints with new intermediate insertions of already present muscles.

The mechanism of annulation in flagellar structures has been studied in detail in the flagellum of the second antennae of isopod crustaceans. *Asellus aquaticus* (Linnaeus, 1758) has been the main species studied, with observations on both normal postembryonic development and regeneration; other species studied have been *Idotea chelipes* (Pallas, 1766), *Lirceus fontinalis* Rafinesque-Schmaltz, 1820 e *Sphaeroma serratum* (Fabricius, 1787). Most of the flagellum of *A. aquaticus* is composed of “quartets”: four articles units where each article has a specific setal distribution pattern. New articles and quartets are produced during the whole life, in the proximal part of the flagellum: the first article divides and produces articles that, relatively independently from each others, divides three more times producing a quartet. During regeneration the mechanism is identical, although there are some difference in the relative development of different quartets, irrespectively of the amputation point. In *L. fontinalis* (Asellidae) most of the flagellum is composed of couples of articles, each one of which bearing setae correspondent to those of two articles of an *A. aquaticus* quartet. The mechanism of production is also very similar, but articles produced by the first one divide just once. In *L. fontinalis* some variability is, anyhow, present and it is sometimes possible to observe three articles units (an article produced by the first one divided twice) and even four article units identical to those of *A. aquaticus*. In *S. serratum* (Sphaeromatidae) most of the flagellum has articles with subequal setal pattern; the mechanism of new article production involves the division of the first article and one further division of the articles produced by it. In *I. chelipes* (Idoteidae) most of the flagellum has articles with subequal setal pattern; the mechanism of new article production involves, unlike *S. serratum*, the division of the first article only.

The mechanism of annulation in flagellar structures and its relationship with growth at the cellular level has been studied in two models (already used for other observations previously described): the flagellum of the second antennae of *A. aquaticus* and the rami of the pleopods of *G. roeselii*. The pleopodal rami of *G. roeselii* increase their article number for the whole life. New articles are produced in the proximal part, by division of the first article only. In this structure, as well as in the second antennal flagellum of *A. aquaticus*, mitotic figures are found only in the proximal part and going distally, to "older" parts, nuclei becomes more spaced and longer. Thus, in both the models studied there is a proximal proliferative zone; cells produced there are then moved distally by the production of new cells and they go through a shape change. How this process is related to the diversity of the segmentation mechanism is not currently understood.

The production of joints and muscle insertions are developmentally correlated processes. Evidence for it was already available for the articles (except tarsomeres) of insect leg, but I have shown in this thesis this is also true for other arthropod appendages, since new joints produced during postembryonic development have also new muscle insertions, if a muscle is present. Joints without any muscle insertion can occur in arthropod appendages, but these have either no muscle passing through or just tendon(s); the occurrence of joints without any muscle insertion but with muscle(s) passing through is currently very doubtful. Thus, the traditional distinction between true article and annuli based on the presence/absence of intrinsic musculature is wrong; articles usually considered annuli may have muscle insertion. Functionally, however, this distinction is still valid, since articles with intermediate insertions of muscles parallel to the proximo-distal axis can not move the appendage independently from other articles as the other true articles (equipped with intrinsic and antagonist muscles confined within them) can do. The naupliar antennal exopod of *Artemia* also provide evidence that joints and muscle insertions are developmentally correlated processes. In this model there are not complete joints, but just "partial" cuticular folds, but also these (which are probably derived from a complete joint) have muscle insertions. A general difference in the timing of expression of true articles and annuli was previously noted and has been here discussed in some deep. A difference in timing exists, but it is not between true articles and annuli (if defined by the presence/absence of muscle insertions) but between articles with independent movements and articles with movements not independent to each others. Also this ontogenetic difference is connected with the different functional morphology of these articles. Flagellar structure also exhibits similarities in their postembryonic development, and these similarities are connected to the presence of a specific proximal "growth zone" (a zone where both new articles are produced and mitoses are localized). The phylogenetic distribution of this growth zone is discussed and it is here proposed to be an ancestral condition for the postembryonic development of (first) antennae and rami of postantennulary appendages of, at least, mandibulate (myriapods, insects and crustaceans) arthropods.

> Fetal apes and early fetal humans have a receding chin, but as the human fetus develops further the chin becomes more prominent. There have been many speculations about why natural selection has favored a protruding chin in humans, making it an exception to the role of neoteny. The answer seems to be, however, not that the chin is specially adaptive but that it does not exist!
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> RICHARD C. LEWONTIN (2000: 77)

INTRODUZIONE

MORFOLOGIA COMPARATA DELLE APPENDICI DEGLI ARTROPODI

Lo zoologo danese Hans Jacob Hansen (1855-1936; si veda WOLFF, 1993 per informazioni generali ed ulteriori riferimenti) scrisse due volumi (HANSEN, 1925, 1930) sulla morfologia comparata delle appendici degli artropodi, risultato di uno studio iniziato più di 30 anni prima (si veda HANSEN, 1893). Fino a quest'oggi quei volumi sono ancora il lavoro più completo e dettagliato scritto da uno stesso autore sulla morfologia delle appendici degli artropodi e questo nonostante non vengano trattati tutti i diversi tipi di appendici per ogni gruppo e non vengano considerati i cambiamenti morfologici durante lo sviluppo postembrionale di tutte le appendici trattate. Da allora le conoscenze su questo argomento sono cresciute enormemente; questo non solo perché nuovi grandi gruppi (come i cefalocaridi ed i remipedi) sono stati scoperti e descritti, ma anche perché nuove tecnologie (come la microscopia elettronica a scansione) hanno permesso di effettuare osservazioni molto più accurate. Inoltre, Hansen non trattò le forme fossili, che ricoprono un ruolo importante nell'attuale discussione sull'evoluzione delle appendici degli artropodi (si vedano KUKALOVÁ-PECK, 1997; WALOSSEK & MÜLLER, 1997; WALOSZEK, 2003; BOXSHALL, 2004; WALOSZEK *et al.*, 2007; ZHANG *et al.*, 2007). Tutto questo illustra chiaramente come la morfologia comparata delle appendici degli artropodi sia un oggetto di studio che necessita una grossa quantità di conoscenze di base.

La morfologia comparata delle appendici degli artropodi, inoltre, presenta ancora molti problemi irrisolti. Uno dei principali limiti per una buona comprensione dell'evoluzione delle appendici risiede nell'attuale mancanza di un consenso sulla filogenesi degli artropodi (una revisione ed un'analisi filogenetica con la più estesa matrice di dati su base sia morfologica che molecolare è presentata da GIRIBET *et al.*, 2005; lavori successivi che portano un importante contributo alla conoscenza della filogenesi degli artropodi sono COOK *et al.*, 2005; REGIER *et al.*, 2005; BEUTEL & POHL, 2006; GLENNER *et al.*, 2006; HARZSCH, 2006; HASSANIN, 2006; MALLATT & GIRIBET, 2006; STOLLEWERK & CHIPMAN, 2006; STRAUSFELD *et al.*, 2006; CARAPELLI *et al.*, 2007). A prescindere dagli effettivi rapporti filogenetice tra i diversi artropodi, però, ci sono ulteriori problemi su diversi aspetti di omologia delle appendici. Solo per fornire alcuni esempi, permangono problemi aperti su: a) l'omologia di alcune parti di appendici tra gruppi diversi (si vedano, per esempio, le discussioni in BITSCH, 2001 e SCHRAM & KOENEMANN, 2001) e talvolta anche tra specie relativamente affini (per esempio, l'attuale classificazione delle branchie dei crostacei brachiuri, che sono modificazioni di una parte d'appendice, è basata su un'effettiva omologia? HONG, 1988); b) l'omologia fra parti simili di appendici differenti di una stessa specie (per esempio, pereiopodi e pleopodi dei crostacei malacostraci, si veda WILLIAMS, 2004); c) la possibile omologia fra appendici diverse di gruppi diversi (per esempio, si veda le similarità tra la prima mascella degli eufausiacei, la seconda mascella dei decapodi ed i toracopodi dei leptostraci discusse in HEEGAARD, 1957). Tutto questo senza menzionare problemi riguardanti strutture specifiche presenti sulle appendici, come il tipo e l'omologia delle sete sensoriali (si veda, per esempio, GARM, 2004 per i crostacei).

In generale, un'appendice di artropode può essere descritta considerando diversi aspetti. Un'appendice può essere stenopodiale (se composta da articoli cilindrici come le zampe di un ragno o d'un insetto), uniramosa, biramosa o poliramosa (se composta da uno, due o più rami). Una distinzione largamente usata tra gli articoli che possono comporre un'appendice è quella tra *articoli veri* ed *annuli*. Questa distinzione è così radicata in morfologia comparata che è spesso esplicitamente descritta anche in testi generali di zoologia degli invertebrati (per esempio, in BRUSCA & BRUSCA, 2003). La differenza tra articoli veri ed annuli si dice essere basata sul sistema muscolare, anche se non è presente un'unica definizione universalmente accettata. Snodgrass definì gli articoli veri come "una qualsiasi parte di appendice con movimenti indipendenti grazie a muscoli inseriti alla sua base in un qualsiasi membro del gruppo degli artropodi" (SNODGRASS, 1935: 85). Aracnologhi (si vedano, per esempio, SHULTZ, 1989; VAN DER HAMMEN, 1989) preferiscono generalmente usare una terminologia differente e, seguendo COUZIJN (1976), fanno riferimento ad articolazioni (o confini articolari) eudesmatiche ed adesmatiche; le prime sono definite come articolazioni in cui almeno un muscolo si inserisce, le seconde come articolazioni in cui non si inserisce alcun muscolo. Una recente definizione recita che "articoli veri sono caratterizzati dalla presenza di muscoli intrinseci che si originano, inseriscono o attaccano all'interno di ogni articolo. Gli annuli, invece, non possiedono origini, inserzioni o punti di attacco muscolari anche se muscoli intrinseci od estensioni tendinee possono passare attraverso degli annuli per inserirsi più distalmente" (BOXSHALL, 2004: 255). Boxshall è stato anche il primo a proporre una precisa distinzione tra annulazioni [annuli] terminali ed intercalari, definendo i primi come articoli completamente privi di muscoli ed i secondi come articoli attraverso i quali uno o più muscoli o tendini passano senza inserirvisi. Le annulazioni terminali, inoltre, sono sempre presenti unicamente nelle porzioni più distali di una parte d'appendice ed un'intera parte composta da annulazioni terminali può essere chiamata flagello terminale (BOXSHALL, 2004). Anche la distribuzione filogenetica degli annuli nelle differenti appendici di diversi artropodi è stata riveduta da BOXSHALL (2004).

SVILUPPO COMPARATO DELLE APPENDICI DEGLI ARTROPODI

In tempi recenti la biologia dello sviluppo comparata è diventata uno strumento molto importante per gli studiosi di evoluzione morfologica (si vedano, per esempio, GOULD, 2002; MINELLI, 2003; CARROLL *et al.*, 2005). Anche se "la ricerca sull'evoluzione dello sviluppo delle appendici degli artropodi è ancora al suo inizio" (PRPIC & DAMEN, 2008: 393), sono già disponibili molti spunti interessanti nati prevalentemente da dati di genetica dello sviluppo comparata (si vedano le revisioni e discussioni in WILLIAMS & NAGY, 1996, 2001; NAGY &

WILLIAMS, 2001; HEMING, 2003; KOJIMA, 2004; WILLIAMS, 2004; ANGELINI & KAUFMAN, 2005; GIORGIANNI & PATEL, 2005; PRPIC & DAMEN, 2008). Il processo di segmentazione (formazione di articoli) delle appendici, ad ogni modo, è stato compreso in modo incompleto perfino per ciò che riguarda le zampe di *Drosophila* (una sintesi è disponibile in KOJIMA, 2004; successivi articoli di interesse sono GALINDO *et al.*, 2005; SAKURAI *et al.*, 2007; TAIJRI *et al.*, 2007).

Per ciò che riguarda la distinzione tra articoli veri ed annuli, il meccanismo di produzione di questi è generalmente considerato come fondamentalmente diverso, il processo di annulazione è generalmente descritto come "secondario" e gli annuli sono generalmente considerati una suddivisione di articoli veri (si vedano, per esempio, SNODGRASS, 1935; MCLAUGHLIN, 1982; BOXSHALL, 2004). Boxshall ha anche notato che "nonostante i dati disponibili da osservazioni sullo sviluppo siano frammentari, è chiaro che gli annuli tendono ad apparire, nello sviluppo di un artropode, successivamente rispetto agli articoli veri" (BOXSHALL, 2004: 292); ciò sembra anche vero per ciò che riguarda la segmentazione delle appendici durante la rigenerazione dove "il numero degli annuli flagellari nel rigenerato aumenta da muta a muta, mentre tutti gli articoli veri sono generalmente già presenti fin dalla prima comparsa del rigenerato" (MARUZZO *et al.*, 2005: 236).

Studi sul moscerino della frutta (*Drosophila*) forniscono effettivamente dati a supporto di una differenza tra il processo di annulazione ed il processo di sviluppo di articoli veri. Nelle appendici di *Drosophila* gli unici articoli definibili come annuli sono quelli più distali delle zampe, i tarsomeri. Il meccanismo molecolare responsabile della formazione dei tarsomeri presenta delle differenze rispetto a quello responsabile per lo sviluppo degli altri articoli della zampa (sintesi in KOJIMA, 2004). Inoltre, rimovendo l'effetto di alcuni geni omeotici sullo sviluppo della zampa si assiste alla produzione di una zampa in cui tutti gli articoli, eccetto i tarsomeri, sono fusi tra loro (CASARES & MANN, 2001; si veda anche PERCIVAL-SMITH *et al.*, 2005). A livello cellulare, mentre i cambiamenti di forma responsabili per la produzione delle articolazioni sono fondamentalmente gli stessi tra tarsomeri ed altri articoli (MIRTH & AKAM, 2002), un recente studio ha dimostrato che l'apoptosi è un processo necessario per una corretta formazione dei soli tarsomeri (MANJÓN *et al.*, 2007).

LO SCOPO DEL PROGETTO

Nel presente studio mi sono concentrato su alcuni aspetti dello sviluppo e dell'anatomia degli articoli delle appendici per capire: a) quali siano, se presenti, le somiglianze nello sviluppo degli annuli di appendici diverse, entro la stessa specie e tra specie di artropodi diversi e b) quali siano, se presenti, le differenze nello sviluppo tra annuli ed articoli veri. A questo scopo ho scelto di indirizzare la ricerca su due aspetti principali: a) il rapporto tra muscoli, inserzioni muscolari ed articolazioni e b) il processo di annulazione nelle strutture flagellari (parti terminali di un'appendice composte unicamente da annuli) ed il suo rapporto con la crescita a livello cellulare.

Secondo le definizioni di articoli veri ed annuli presentate precedentemente, l'anatomia del sistema muscolare è l'aspetto più importante. Un generico articolo vero di un'appendice di un artropode mandibolato possiede un paio di muscoli antagonisti le cui inserzioni terminali sono direttamente connesse alla placca cuticolare dell'articolo seguente, ovvero l'inserzione dei muscoli è sull'articolazione; questa descrizione corrisponde alla tipica articolazione "pivotale" descritta da MANTON (1977). Per questo tipo di articoli ci sono dati a supporto di uno stretto legame nello sviluppo delle cellule della membrana artrodiale (le cellule epidermiche che producono l'articolazione) e delle inserzioni muscolari. Il più antico dato risale probabilmente ad un articolo di Child e Young i quali, studiando la rigenerazione delle zampe di alcune libellule, notarono che l'assenza di articolazioni ben formate era direttamente correlabile con l'assenza di una normale inserzione muscolare (CHILD & YOUNG, 1903). Nonostante non sia chiaro se le cellule epidermiche che mediano l'inserzione muscolare (le cosiddette cellule tendinee) siano anche cellule della membrana artrodiale, è già stato notato nelle zampe di *Drosophila* che il meccanismo molecolare responsabile del differenziamento di questi tipi cellulari è, almeno in parte, simile (SOLER *et al.*, 2004). Nelle zampe degli insetti lo sviluppo degli apodemi (invaginazioni cuticolari delle articolazioni) è strettamente correlato con lo sviluppo dei muscoli (BALL *et al.*, 1985) e sopprimendo, tramite microchirurgia, lo sviluppo degli apodemi si interferisce con il corretto differenziamento dei muscoli che vi si sarebbero dovuti inserire (FOURNIRE, 1968). Tutti questi dati mostrano che lo sviluppo delle articolazioni, dei muscoli e delle inserzioni muscolari possono essere processi strettamente legati, anche se una certa variazione nel legame reciproco tra questi è attesa, dato che gli annuli sono considerati, come detto sopra, articolazioni senza alcuna inserzione muscolare.

Le strutture flagellari mostrano spesso un incremento indeterminato nel numero di annuli nel corso dello sviluppo postembrionale. Il meccanismo tramite il quale vengono prodotti nuovi annuli è stato studiato solo in poche specie ed unicamente per il flagello antennale (si veda IMMS, 1940 per ciò che riguarda le antenne degli insetti). Nei pochi lavori dove sono stato studiati sia il meccanismo di produzione di nuovi articoli che la crescita, è stato notato uno stretto legame (HAAS, 1955; CAMPBELL & PRIESTLEY, 1970; MARUZZO, 2003), ma solo HAAS (1955) ha studiato anche aspetti di dinamiche cellulari. Un approccio comparativo focalizzato a livello cellulare risulta inoltre particolarmente desiderabile, in quanto gli aspetti di sviluppo a livello cellulare sono attualmente quelli meno noti per le appendici degli artropodi. Esiste un'enorme quantità di lavori che descrivono cambiamenti morfologici durante lo sviluppo (anche se la maggior parte di questi sono descrizioni di stadi larvali o giovanili ai fini del riconoscimento delle specie) e dati di genetica dello sviluppo stanno aumentando molto rapidamente. A livello cellulare, però, sono disponibili pochi lavori; perfino per ciò che riguarda le appendici di *Drosophila*, uno degli animali più studiati in assoluto, alcuni importanti aspetti dello sviluppo delle sue appendici a livello cellulare sono stati chiariti solo in tempi molto recenti (si vedano HE & ADLER, 2001 per le antenne; MIRTH & AKAM, 2002 e TAYLOR & ADLER, 2008 per le zampe; BAENA-LÓPEZ *et al.*, 2005 e TAYLOR & ADLER, 2008 per le ali). Per quanto riguarda artropodi non insetti, sono disponibili specifici studi a livello cellulare sullo sviluppo delle appendici sono per i fillopodi di crostacei branchiopodi (si vedano FREEMAN *et al.*, 1992; WILLIAMS & MÜLLER, 1996; FREEMAN, 2005; WILLIAMS, 2007). Questa mancanza di conoscenza a livello cellulare è un forte limite anche per la genetica dello sviluppo, dato che risulta difficile interpretare dati di espressione genica senza un'adeguata comprensione della morfogenesi dell'appendice e della sua variazione evolutiva (WILLIAMS, 2004).

GLI STUDI EFFETTUATI

Il presente studio è concentrato su due aspetti principali: a) il rapporto tra muscoli, inserzioni muscolari ed articolazioni e b) il processo di annulazione nelle strutture flagellari ed il suo rapporto con la crescita a livello cellulare. Per studiare entrambi questi aspetti sono stati utilizzati diversi modelli e diverse tecniche. La presente tesi è scritta come collezione di articoli; in questa sezione spiegherò il tipo di osservazioni effettuate, nella sezione successiva descriverò brevemente i diversi manoscritti che compongono la presente tesi.

A) IL RAPPORTO TRA MUSCOLI, INSERZIONI MUSCOLARI ED ARTICOLAZIONI

Le appendici naupliari. Le appendici dei nauplii sono state scelte come modello principale per lo studio del rapporto tra muscoli, inserzioni muscolari ed articolazioni. Il nauplio è il tipo più comune di larva dei crostacei (per una breve sintesi si veda DAHMS *et al.*, 2006; sintesi che deve essere aggiornata con la recente scoperta di stadi naupliari anche nei crostacei remipedi, si veda KOENEMANN *et al.*, 2007). I nauplii sono definiti dal possedere solo tre paia di appendici funzionali: antenne prime (o antennule; sempre uniramose), antenne seconde (generalmente biramose) e mandibole (generalmente biramose). Mentre esiste una vasta letteratura descrittiva sulla morfologia esterna dei diversi stadi naupliari per tutti i maggiori gruppi di crostacei, sono attualmente disponibili solo poche descrizioni dell'anatomia interna e, tra queste, quelle che descrivono la muscolatura intrinseca delle appendici sono una rarità. In questo studio ho focalizzato la mia attenzione sulle antenne prime e sul ramo esterno (esopodite) delle antenne seconde e delle mandibole, perché è in queste strutture che è presente una maggiore diversità, sia ontogenetica che filogenetica, nel numero di articoli.

La letteratura sull'articolazione delle appendici naupliari ed il loro sistema muscolare è riveduta in dettaglio. Due specie, appartenenti a due gruppi per cui non erano disponibili dettagliate osservazioni sulla muscolatura intrinseca delle appendici naupliari, sono state prese in particolare considerazione. Una delle specie da me studiate è *Balanus improvisus* Darwin, 1854 (Crustacea, Cirripedia), scelto in quanto: a) è relativamente facile reperirne i nauplii; b) era già noto il fatto che l'esopodite delle antenne seconde aumenta il numero di articoli durante lo sviluppo naupliare (KADO & HIRANO, 1994); c) marcature con fallotossine ed anticorpi erano già state usate con successo nei nauplii di questa specie (SEMMLER, 2005; SEMMLER *et al.*, 2006). L'altra specie studiata, *Artemia* sp. (Crustacea, Branchiopoda), è stata scelta in quanto: a) anche se uno dei crostacei più usati come modello di studio, ancora poco era noto sulla morfologia esterna e sull'anatomia interna delle sue appendici naupliari; b) marcature con fallotossine ed anticorpi erano già state usate con successo nei nauplii di questa specie (si veda, per esempio, CRIEL & MACRAE, 2002). Solo l'esopodite delle antenne seconde è stato studiato nei nauplii di *Artemia*, questo perché le prime antenne di questa specie non sono divise in articoli e le mandibole sono uniramose, con il solo ramo interno (endopodite) presente. La morfologia esterna è stata studiata tramite microscopia in campo chiaro,microscopia elettronica a scansione e tramite una recente tecnica che prevede l'uso della microscopia confocale a scansione laser; il sistema muscolare è stato studiato tramite marcature con falloidina ed anticorpi contro α-tubulina. I risultati di queste osservazioni sono presentati e discussi nel *manoscritto 4* e nel *manoscritto 5*.

Appendici con muscoli decorrenti parallelamente all'asse prossimo-distale. Preliminari osservazioni sulle appendici naupliari hanno rivelato che articoli generalmente considerati annuli possedevano in realtà inserzioni intermedie di muscoli decorrenti parallelamente all'asse prossimo-distale (si veda *manoscritto 5*); a seguito di questa inaspettata osservazione ho deciso di verificare l'effettiva differenza anatomica tra una parte di appendice generalmente descritta come composta da articoli veri ed una generalmente descritta come composta da annuli e per la quale, per entrambi i modelli, fosse nota la presenza di muscoli decorrenti lungo l'intera parte, parallelamente all'asse prossimo-distale.

Le antenne del miriapode *Lithobius forficatus* (Linnaeus, 1758) (Chilopoda, Lithobiomorpha) sono state uno dei due modelli utilizzati; la presenza di quattro muscoli decorrenti lungo tutta l'antenna parallelamente all'asse prossimo-distale e la presenza di inserzioni muscolari intermedie in corrispondenza di ogni articolazione attraversata da questi muscoli era già nota (IMMS, 1939). Il secondo modello scelto sono stati i pleopodi del malacostraco *Gammarus roeselii* Gervais, 1835 (Crustacea, Amphipoda). In *G. roeselii*, come regola per gli anfipodi gammaridei, le prime tre paia di appendici (pleopodi) dell'addome (pleon) sono biramose, con una porzione prossimale portante due rami generalmente descritti come annulati. La presenza di pochi muscoli (due in *Eurythenes gryllus* (Lichtenstein, 1887), BOUDRIAS, 2002) decorrenti lungo l'intera lunghezza dei rami era già nota, ma non erano disponibili studi specifici sulle inserzioni muscolari. In entrambi questi modelli, inoltre, si registra un aumento del numero di articoli durante tutto lo sviluppo postembrionale. Lo studio è stato eseguito tramite sezioni di esemplari inclusi in paraffina e colorati con ematossilina-eosina. I risultati di questo studio sono presentati e discussi nel *manoscritto 6*.

B) IL PROCESSO DI ANNULAZIONE NELLE STRUTTURE FLAGELLARI ED IL SUO RAPPORTO CON LA CRESCITA A LIVELLO CELLULARE

Produzione di nuovi articoli nel flagello delle seconde antenne di Asellus aquaticus *ed altri isopodi*. Il flagello delle seconde antenne di *Asellus aquaticus* (Linnaeus, 1758) (Crustacea, Isopoda) è composto da annuli (il flagello è completamente privo di muscoli; WEGE, 1911) il cui numero aumenta durante tutta la vita dell'animale. Il meccanismo grazie a cui nuovi annuli sono prodotti era già stato studiato con un certo dettaglio sia durante lo sviluppo normale (RACOVITZA, 1925; MARUZZO, 2003; RONCO, 2004) che durante la rigenerazione (MARUZZO, 2003). Nel presente studio ho completato i lavori precedenti con osservazioni sia di microscopia ottica che di microscopia elettronica a scansione. Per meglio comprendere l'evoluzione del meccanismo di produzione degli articoli ho inoltre studiato il flagello delle seconde antenne degli isopodi *Idotea chelipes* (Pallas, 1766), *Lirceus fontinalis* Rafinesque-Schmaltz, 1820 e *Sphaeroma serratum* (Fabricius, 1787). I risultati di questo studio sono presentati e discussi nel *manoscritto 1*, *manoscritto 2* e *manoscritto 3*.

Produzione di nuovi annuli e crescita a livello cellulare. Il rapporto tra produzione di nuovi annuli e crescita a livello cellulare (divisione e cambiamento di forma) è stato studiato in due strutture che aggiungono nuovi articoli, generalmente descritti come annuli, durante tutta la vita: il flagello delle antenne seconde di *Asellus aquaticus* ed i rami dei pleopodi di *Gammarus roeselii*. Il flagello delle antenne seconde di *A. aquaticus* è stato scelto come modello di struttura completamente priva di muscoli, i rami dei pleopodi di *G. roeselii* come struttura con muscoli. La morfologia esterna è stata studiata tramite microscopia ottica e microscopia elettronica a scansione mentre le dinamiche dell'epidermide sono state indagate tramite marcature nucleari. I risultati di questo studio sono presentati e discussi nel *manuscritto 7*.

DESCRIZIONE DEI MANOSCRITTI

MANOSCRITTO 1

TITOLO: Crescita e rigenerazione delle antenne seconde di *Asellus aquaticus* (Isopoda) nel contesto della segmentazione delle antenne negli artropodi.
AUTORI: Diego Maruzzo, Alessandro Minelli, Monica Ronco e Giuseppe Fusco.
STATO: pubblicato nel *Journal of Crustacean Biology*, vol. 27(2), pag. 184-196 (2007).

Questo manoscritto descrive in dettaglio il meccanismo di produzione di nuovi articoli nel flagello delle antenne seconde di *A. aquaticus*, sia durante il normale sviluppo postembrionale che a seguito di amputazioni a livello del punto preferenziale di rottura delle antenne. La maggior parte dei dati su cui questo manoscritto è basato sono il prodotto di una precedente tesi di dottorato (RONCO, 2004) e della mia tesi di laurea (MARUZZO, 2003). Il manoscritto è stato comunque incluso nella presente tesi perché la discussione presente in questo, che fornisce le basi teoriche su cui la maggior parte dei seguenti manoscritti di questa tesi si fondano, è il prodotto di un lavoro di elaborazione teorica compiuto durante il mio dottorato. Nei paragrafi "sviluppo comparato delle antenne degli isopodi" e "struttura e sviluppo comparato delle antenne degli artropodi", le similarità nel meccanismo di produzione degli articoli nelle antenne sono discusse con un certo dettaglio; queste discussioni forniscono un'importante base teorica per il *manoscritto 3* e per il *manoscritto 7*. Nel paragrafo "sviluppo degli annuli versus "articoli veri"", particolarità dello sviluppo, soprattutto rispetto allo sviluppo relativo, degli annuli e degli articoli veri sono discusse in dettaglio, prevalentemente espandendo la discussione di BOXSHALL (2004); questa discussione fornisce un'importante base teorica per il *manoscritto 5* e per il *manoscritto 6*.

Contributo degli autori: DM ha pianificato lo studio, svolto la maggior parte degli esperimenti, interpretato i dati, elaborato la parte teorica e scritto il testo; AM ha ideato lo studio, revisionato il contenuto intellettuale e partecipato alla stesura del testo; MR ha ideato e pianificato lo studio, svolto alcuni esperimenti ed interpretato i dati; GF ha revisionato il contenuto intellettuale e partecipato alla stesura del testo.

MANOSCRITTO 2

TITOLO: Formazione del pattern segmentale a seguito di amputazioni nel flagello delle antenne seconde di *Asellus aquaticus* (Crustacea, Isopoda).
AUTORI: Diego Maruzzo, Mia Egredzija, Alessandro Minelli e Giuseppe Fusco.
STATO: in pubblicazione nell'*Italian Journal of Zoology*.

Il manoscritto descrive in dettaglio la produzione di nuovi articoli nel flagello delle antenne seconde di *A. aquaticus* a seguito di amputazioni lungo il flagello stesso. La maggior parte dei dati su cui il manoscritto è basato sono il prodotto di due tesi di laurea (MARUZZO, 2003; EGREDZIJA, 2006). Questo lavoro completa i dati del manoscritto precedente permettendo una comprensione più completa del meccanismo di produzione degli articoli durante la rigenerazione nelle antenne seconde di *A. aquaticus*.

Contributo degli autori: DM ha pianificato lo studio, svolto esperimenti, interpretato i dati, elaborato la parte teorica e scritto il testo; ME ha svolto esperimenti ed interpretato i dati; AM e GF hanno revisionato il contenuto intellettuale e partecipato alla stesura del testo.

MANOSCRITTO 3

TITOLO: Morfologia comparata e sviluppo delle antenne seconde degli isopodi (Crustacea).
STATO: manoscritto non pubblicato.

Questo manoscritto descrive la morfologia ed il meccanismo di produzione di nuovi articoli durante lo sviluppo postembrionale delle antenne seconde degli isopodi *Idotea chelipes*, *Lirceus fontinalis* and *Sphaeroma serratum*. Dato che queste osservazioni sono state compiute sia con microscopia ottica che con microscopia elettronica a scansione (SEM) e poiché per *Asellus aquaticus*, principale termine di confronto, non erano disponibili osservazioni tramite SEM, queste sono state prodotte anche per questa specie. Questo lavoro completa la discussione iniziata nel *manoscritto 1* fornendo dati originali utili per comprendere l'evoluzione sia della morfologia del flagello che del meccanismo di formazione di nuovi annuli durante lo sviluppo postembrionale negli isopodi.

MANOSCRITTO 4

TITOLO: Discrepanza segmentale nelle appendici di artropodi: l'esopodite dell'antenne naupliare di *Artemia* (Crustacea, Branchiopoda, Anostraca).
AUTORI: Diego Maruzzo, Alessandro Minelli e Giuseppe Fusco.
STATO: in avanzato stato di preparazione per invio ad *Arthropod Structure & Development*.

Questo manoscritto descrive la morfologia esterna ed il sistema muscolare dell'esopodite delle antenne nei nauplii di *Artemia* sp. In questo modello, non sono presenti articolazioni complete. Questa inedita osservazione ha fornito l'opportunità di rivedere e discutere la presenza di simili casi di discrepanza segmentale nelle appendici di artropodi. Particolare attenzione è stata posta al rapporto tra inserzioni dei muscoli e pieghe cuticolari.

Contributo degli autori: DM ha pianificato lo studio, svolto gli esperimenti, interpretato i dati, elaborato la parte teorica e scritto il testo; AM e GF hanno revisionato il contenuto intellettuale e partecipato alla stesura del testo.

MANOSCRITTO 5

TITOLO: Articoli e muscolatura nelle appendici naupliari.
STATO: manoscritto non pubblicato.

Questo manoscritto fornisce una sintesi sulla variazione ontogenetica del numero di articoli e della struttura del sistema muscolare nelle appendici naupliari. Il sistema muscolare delle appendici naupliari di un cirripede (*Balanus improvisus*), inoltre, è qui descritto per la prima volta. I dati sono successivamente discussi in modo comparato per capire, seguendo la discussione del *manoscritto 1*, le differenze ontogenetiche tra articoli veri ed annuli.

MANOSCRITTO 6

TITOLO: Sull'anatomia di alcuni articoli delle appendici degli artropodi
STATO: manoscritto non pubblicato.

Questo manoscritto descrive la muscolatura, con particolare attenzione alle inserzioni muscolari, delle antenne del centopiedi *Lithobius forficatus* e dei pleopodi dell'anfipode *Gammarus roeselii*. La base anatomica della distinzione tra articoli veri ed annuli viene, quindi, discussa alla luce di questi dati nonché delle conclusione del precedente manoscritto.

MANOSCRITTO 7

TITOLO: Sulla "zona di crescita" prossimale delle appendici di artropodi.
STATO: manoscritto non pubblicato.

Questo manoscritto descrive il meccanismo di produzione di nuovi articoli durante lo sviluppo postembrionale nei rami dei pleopodi dell'anfipode *Gammarus roeselii*, nonché lo sviluppo dell'epidermide in questo stesso modello e nel flagello delle antenne seconde di *Asellus aquaticus*. Il significato di una specifica "zone di crescita" prossimale nelle appendici degli artropodi viene definito e la distribuzione filogenetica di una zona con tali caratteristiche è inoltre discussa.

BIBLIOGRAFIA

Si veda “references” a pag. 27-30.

> Fetal apes and early fetal humans have a receding chin, but as the human fetus develops further the chin becomes more prominent. There have been many speculations about why natural selection has favored a protruding chin in humans, making it an exception to the role of neoteny. The answer seems to be, however, not that the chin is specially adaptive but that it does not exist!
>
> RICHARD C. LEWONTIN (2000: 77)

INTRODUCTION

COMPARATIVE MORPHOLOGY OF ARTHROPOD APPENDAGES

The Danish zoologist Hans Jacob Hansen (1855-1936; see WOLFF, 1993 for basic information and further references) devoted two volumes (HANSEN, 1925, 1930) to the comparative morphology of arthropod appendages. These volumes are the summary of a work started more than 30 years before (see HANSEN, 1893). Up to now these volumes are still the more comprehensive and detailed summary of the of morphology of arthropod appendages written by one author, nevertheless they did not deal with all the appendages for all the groups or with the postembryonic changes of all the appendages described. Knowledge has grown enormously since then, not only because more species and also major groups (e.g., cephalocarids and remipedes) have been discovered and described, but also because new techniques (such as scanning electron microscopy) have allowed to perform much more accurate observations. Also, Hansen did not deal with fossils, and fossils play and important role in the current discussion about arthropod appendage evolution (e.g., KUKALOVÁ-PECK, 1997; WALOSSEK & MÜLLER, 1997; WALOSZEK, 2003; BOXSHALL, 2004; WALOSZEK *et al.*, 2007; ZHANG *et al.*, 2007). All this just to explain that arthropod appendage comparative morphology is a subject that must be dealt with a huge amount of background knowledge.

Comparative morphology of arthropod appendages is also a subject marred with problems. One of the main limits for an understanding arthropod appendages evolution is that, currently, there is no widely accepted arthropod phylogeny (a review of the subject as well as a phylogenetic analysis with the bigger matrix, with both molecular and morphological data, is GIRIBET *et al.*, 2005; more recent papers relevant for arthropod phylogeny are COOK *et al.*, 2005; REGIER *et al.*, 2005; BEUTEL & POHL, 2006; GLENNER *et al.*, 2006; HARZSCH, 2006; HASSANIN, 2006; MALLATT & GIRIBET, 2006; STOLLEWERK & CHIPMAN, 2006; STRAUSFELD *et al.*, 2006; CARAPELLI *et al.*, 2007). Anyway, irrespectively of the actual phylogenetic relationships of different arthropod groups, there are still problems with different aspects of homology of arthropod appendages. Just to give some examples, these problems involve: a) the homology of some specific parts of homologous appendages in different groups (see discussion in, e.g., BITSCH, 2001 and SCHRAM & KOENEMANN, 2001) and sometimes also for relatively close related species (e.g., is the current classification of brachyuran crustacean gills, which are modification of appendage outgrowths, based on proper homology? HONG, 1988); b) the homology of similar parts of different appendages within the same species (e.g., pereiopods and pleopods of malacostracan crustaceans, see WILLIAMS, 2004); c) the possible homology of different appendages of different groups (see, e.g., the similarities between the first maxilla of euphausiids, the second maxilla of decapods and the thoracopods of leptostracans, discussed in HEEGAARD, 1957). All of these not to mention problems with specific structures present on the appendages, such as sensory setae (see, e.g., GARM, 2004 for crustaceans).

In general, an arthropod appendage may be described by taking into account different aspects. An appendage can be stenopodial (if made of cylindrical articles, as in a spider or insect legs) or phyllopodial (if more or less leaf-shaped), uniramous, biramous or polyramous (if made of one, two or more rami). A widely used distinction among articles is the one between *true articles* and *annuli*. This distinction is so used in comparative morphology that is even explicitly stated in invertebrate zoology textbooks (e.g., BRUSCA & BRUSCA, 2003). The difference between true articles and annuli is often claimed to be based on the anatomy of the muscular system, however there is no clear, widely accepted definition. Snodgrass defined true articles as "any part of an appendage independently movable in some member of the Arthropoda by muscles inserted on its base" (SNODGRASS, 1935: 85). Students of arachnids (e.g., SHULTZ, 1989; VAN DER HAMMEN, 1989) use a slightly different terminology and, following COUZIJN (1976), refer to eudesmatic and adesmatic joints (or article boundaries), with the former defined as joints where at least one muscle insert, and the latter with no muscle insertion. One recent definition states that "true segments [articles] are characterised by the presence of intrinsic muscles that originate, insert, or attach within each segment [article]. By contrast, annuli lack intrinsic muscle origins, intermediate attachments or insertions, although intrinsic muscles or their tendinous extensions may pass through annuli to a more distal insertion site" (BOXSHALL, 2004: 255). Boxshall also proposes a distinction between terminal and intercalary annulations [annuli], considering terminal annulations the articles devoid of any muscle and intercalary annulations those with muscle(s) or tendon(s) passing through. Terminal annulations are always present in the distalmost part of appendages and the whole structure with terminal annulations may be called terminal flagella (BOXSHALL, 2004). The phylogenetic distribution of annuli in different appendages has been reviewed by BOXSHALL (2004).

COMPARATIVE DEVELOPMENT OF ARTHROPOD APPENDAGES

Recently, comparative developmental biology has become a very important topic for students of morphological evolution (e.g., GOULD, 2002; MINELLI, 2003; CARROLL *et al.*, 2005). Although "research into the evolution of arthropod appendage development is still very much at its beginning" (PRPIC & DAMEN, 2008: 393), there are several interesting insights, mainly from comparative developmental genetics (see review and discussion in WILLIAMS & NAGY, 1996, 2001; NAGY & WILLIAMS, 2001; HEMING, 2003; KOJIMA, 2004; WILLIAMS, 2004; ANGELINI & KAUFMAN, 2005; GIORGIANNI & PATEL, 2005; PRPIC & DAMEN, 2008). However, the process of segmentation (formation of articles) is only starting to be understood even in *Drosophila* legs (review in KOJIMA, 2004; subsequent papers of interest are GALINDO *et al.*, 2005; SAKURAI *et al.*, 2007; TAIJRI *et al.*, 2007).

Concerning the distinction between true articles and annuli in arthropod appendages, the formation of true articles and annuli have often been regarded as different processes, with the annulation process often described as "secondary" and annuli considered as a subdivision of a true article (e.g., SNODGRASS, 1935; MCLAUGHLIN, 1982; BOXSHALL, 2004). Boxshall noted that "although data from available developmental studies are somewhat fragmentary, it is apparent that annuli tend to appear later in arthropod development than limb segments [true articles]" (BOXSHALL, 2004: 292); this seems also true for the process of segmentation during the regeneration of an appendage where "the number of flagellar units [annuli] in the regenerate increases from moult to moult, whereas all 'true segments' [true articles] are usually formed as soon as the regenerate comes out first" (MARUZZO *et al*., 2005: 236).

Studies from the fruit fly also provide evidence that annulation is a different process. In *Drosophila* appendages the only articles that can be classified as annuli are the distalmost ones in the leg, i.e., the tarsomeres. Molecular mechanisms involved in tarsus patterning show peculiar differences in respect to those producing the remaining leg articles (reviewed in KOJIMA, 2004). Also, removing homeotic-selector gene influence from leg development causes the production of a leg with all its segments fused together, but for the tarsomeres, which are normally produced (CASARES & MANN, 2001; see also PERCIVAL-SMITH *et al.*, 2005). At the cellular level, while the cell shape changes involve in joint production are the basically the same between tarsomeres and other articles (MIRTH & AKAM, 2002), recent findings show that apoptosis is required for proper formation of tarsomeres only (MANJÓN *et al*., 2007).

THE PURPOSE OF THE PROJECT

In the present project I studied selected aspects of article anatomy and development, in order to understand: a) which are (if present) the developmental similarities among annuli of different appendages and different arthropods, and b) which are (if present) the developmental differences between annuli and true articles. I decided to focalise the research on two topics: a) the relationships between muscles, muscle insertions and joints, and b) the mechanism of annulation in flagellar structures (terminal part of an appendage composed of only annuli) and its relationship with growth at the cellular level.

According to the definitions of true articles and annuli given above, the anatomy of the muscular system is the most important aspect. A generalized true article of a mandibulate arthropod leg has a pair of antagonistic muscles of which the distal insertions are directly connected to the sclerites of the following article, i.e. muscle insertions are on the joint (or article boundary), as in the typical pivot joint described by MANTON (1977). For these articles there is evidence of a close developmental relationship between the development of the arthrodial membrane cells (epidermal cells that produce the joint) and muscle insertions. The oldest evidence is probably to be found in a paper by Child and Young who, while working on the regeneration of damselfly leg, noted that the absence of a proper joint might be directly due to the absence of the proper muscle insertion (CHILD & YOUNG, 1903). While it is not clear if the epidermal cell mediating muscle insertion (tendinal cell) are also arthrodial membrane cells, it has already been noted that the molecular mechanism involved in the differentiation of both of these in *Drosophila* is partially similar (SOLER *et al.*, 2004). In insect leg the development of the apodemes (cutuclar invaginations of the joints) is strictly related with the development of the muscle (BALL *et al.*, 1985), and suppressing by microsurgery the development of apodemes, interferes with the differentiation and segregation of the muscles that would insert there (FOURNIRE, 1968). This evidence clearly shows that the development of joint, muscle, and muscle insertion can be developmentally correlated processes, however some variation is expected as annuli are supposed to be joint without any muscle insertion.

Flagellar structures often show indeterminate post-embryonic increase in the number of annuli. The mechanism by which new annuli are produced has been studied only in few species or groups, and only for the antennal flagellum (see IMMS, 1940 for insect antennae). In the few works where both the mechanism of article production and the overall growth have been studied, a close relationship between the two was noted (HAAS, 1955; CAMPBELL & PRIESTLEY, 1970; MARUZZO, 2003) but only HAAS (1955) studied it also at the cellular level. A comparative developmental approach focussed at the cellular level is also desirable because this is the level for which less data are available about arthropod appendages. There is a huge amount of papers containing information on the changes of the external morphology (although most of them are focussed on taxonomic description of larval or juvenile stages), and data on developmental genetics are growing very rapidly. At the cellular level, however, there are very few works; even for the appendages of *Drosophila*, one of the most widely studied model animals, some aspects of the development of its appendages at the cellular level have been elucidated only in the last few years (see HE & ADLER, 2001 for the antennae; MIRTH & AKAM, 2002 and TAYLOR & ADLER, 2008 for the legs; BAENA-LÓPEZ *et al.*, 2005 and TAYLOR & ADLER, 2008 for the wings), and concerning non-insect arthropods there are specific studies on cellular aspects of appendage development only for the phyllopods of branchiopod crustacean (FREEMAN *et al.*, 1992; WILLIAMS & MÜLLER, 1996; FREEMAN, 2005; WILLIAMS, 2007). This lack is also a strong limit for developmental genetics because it is difficult to interpret gene expression data without an adequate understanding of appendage morphogenesis and its evolutionary variation (WILLIAMS, 2004).

THE PERFORMED STUDIES

The present study has two main focuses: a) the relationship between muscles, muscle insertions and joints, and b) the mechanism of annulations in flagellar structures and its relationship to growth at the cellular level. These two aspects were studied in different models and with different techniques. The present thesis is written as a paper collection; in this section I first explain what kind of observations I decided to perform, later I give a brief overview of the manuscripts that compose this thesis.

A) THE RELATIONSHIP BETWEEN MUSCLES, MUSCLE INSERTIONS AND JOINTS

The naupliar appendages. The appendages of nauplii have been chosen as main model for studying the relationship between muscles, muscle insertions and joints. The nauplius is the most common type of larva in crustaceans (review in DAHMS *et al.*, 2006; a review to be updated with the recent discovery of naupliar stages also in remipede crustaceans, see KOENEMANN *et al.*, 2007); crustacean nauplii are diagnosed by the possess of only three pairs of functional appendages: first antennae [or antennule] (always uniramous), second antennae (usually biramous) and mandibles (usually biramous). While there is a huge amount of descriptions of the external morphology in the different naupliar stadia for all the major crustacean groups, there are just few descriptions of the internal anatomy, and, among these, only few described the intrinsic musculature of the appendages. In this study I focussed on the first antennae and on the outer ramus (the exopod) of the second antennae and mandibles because it is in these structures that there is more diversity, both ontogenetical and phylogenetical, in article number.

The literature on articulation of naupliar appendages and their muscular system has been extensively reviewed. In addition, two species, belonging to two groups for which no detailed account on the naupliar appendages muscular system was available, have been directly studied. The first specie studied is *Balanus improvisus* Darwin, 1854 (Crustacea, Cirripedia) and it was chosen because: a) rather easy to obtain nauplii; b) it was already known that the second antennal exopod increases in article number during naupliar development (KADO & HIRANO, 1994); c) phallotoxin and antibody staining were already used in this animal successfully (SEMMLER, 2005; SEMMLER *et al.*, 2006). The other specie studied, *Artemia* sp. (Crustacea, Branchiopoda), was chosen because: a) even if it is one of the most used models among crustaceans, little is still known about the external and internal anatomy of the naupliar appendages; b) phallotoxin and antibody staining were already used in this animal successfully (see, e.g., CRIEL & MACRAE, 2002). Only the second antennal exopod of *Artemia* nauplia was studied because the first antennae are unsegmented and the mandibles are uniramous, with the only inner ramus (endopod) present. The external morphology was studied with both light and scanning electron microscopy and with a new technique involving the use of confocal laser scanning microscopy, the muscular system was studied with phalloidin and antibody against α-tubulin staining; data of these observation are shown and discussed in *manuscript 4* and *manuscript 5*.

Appendages with muscles running parallel to the proximo-distal axis. Early observations on naupliar appendages revealed that articles generally described as annuli indeed have intermediate muscle insertions of muscle(s) running parallel to the proximo-distal axis (see *manuscript 5*); accordingly, I decided to check the actual anatomical difference between a part of appendage generally described as made of true articles and one generally described as made of annuli, by selecting two arthropods for both of which it was already known there were just few muscles running throughout the part, parallel to the proximo-distal axis.

The antennae of the myriapod *Lithobius forficatus* (Linnaeus, 1758) (Chilopoda, Lithobiomorpha) were chosen as one model; it was already known that there are four muscles running throughout the antenna parallel to the proximo-distal axis, and these muscles have intermediate insertions on each joint they pass through (IMMS, 1939). As second model I chose the pleopods of the malacostracan *Gammarus roeselii* Gervais, 1835 (Crustacea, Amphipoda). *G. roeselii* has, as rule for gammaridean amphipods, the first three pairs of appendages (pleopods), of the abdomen (pleon) biramous, with a proximal common section and two rami generally described as annulated; there are few muscles (two in *Eurythenes gryllus* (Lichtenstein, 1887), BOUDRIAS, 2002) running throughout the rami, but there is no specific study on the insertion pattern of them. Both of these models also add new articles during the whole postembryonic development. These two models were studied through paraffin section stained with haematoxylin-eosin; data of these observations are shown and discussed in *manuscript 6*.

B) THE MECHANISM OF ANNULATIONS IN FLAGELLAR STRUCTURES AND ITS RELATIONSHIP WITH GROWTH AT THE CELLULAR LEVEL

Production of new annuli in second antennal flagellum of Asellus aquaticus *and other isopods*. The second antennal flagellum of *Asellus aquaticus* (Linnaeus, 1758) (Crustacea, Isopoda) is composed of annuli (the flagellum is completely devoid of muscles; WEGE, 1911) whose number increases during the whole life of the animal. The mechanism by which new annuli are produced was already described to some extent during both normal development (RACOVITZA, 1925; MARUZZO, 2003; RONCO, 2004) and regeneration (MARUZZO, 2003). Here I completed the previous studies by making further observations with light microscopy as well as scanning electron microscopy. To investigate the evolution of the mechanism of article production I also studied the second antennal flagellum of the isopods *Idotea chelipes* (Pallas, 1766), *Lirceus fontinalis* Rafinesque-Schmaltz, 1820 and *Sphaeroma serratum* (Fabricius, 1787). Data on these observations are shown and discussed in *manuscript 1*, *manuscript 2* and *manuscript 3*.

Relationship between production of new annuli and growth at the cellular level. The relationship between production of new annuli and growth at the cellular level (division and shape change) was studied in two structure that keeps on adding new articles, generally described as annuli, during the whole life: the second antennal flagellum of *Asellus aquaticus* and the rami of the pleopods of *Gammarus roeselii*. The second antennal flagellum of *A. aquaticus* was chosen as a model of an appendage section completely devoid of muscles, the pleopods of *G. roeselii* as an appendage section with muscles. The external morphology was studied with light and scanning electron microscopy and the dynamics of the epidermis was studied with nuclear staining; data of these observations are shown and discussed in *manuscript 7*.

DESCRIPTION OF THE MANUSCRIPTS

MANUSCRIPT 1

TITLE: Growth and regeneration of the second antennae of *Asellus aquaticus* (Isopoda) in the context of arthropod antennal segmentation.
AUTHORS: Diego Maruzzo, Alessandro Minelli, Monica Ronco and Giuseppe Fusco.
STATUS: published in the *Journal of Crustacean Biology*, vol. 27(2), pp. 184-196 (2007).

This manuscript describes in detail the production of new articles in the flagellum of the second antennae of *A. aquaticus* during normal postembryonic development as well as following amputation at the preferred breakage point. Most of the data on which the paper is based are the product of an earlier PhD thesis (RONCO, 2004) and on my own master thesis (MARUZZO, 2003). The manuscript has been anyway included in this PhD thesis because the discussion developed in it, which includes the theoretical background for most of the further manuscripts, is the product of the early theoretical work carried out during my PhD work. In the paragraphs "comparative development of isopod antennae" and "comparative structure and development of arthropod antennae" general similarities in the mechanism of article production in the antennae are discussed; these discussions are an important theoretical foundation for manuscript 3 and manuscript 7. In the paragraph "development of annulations versus "true" articles" the developmental base, especially the relative timing, of annuli and true articles is discussed, mainly expanding the discussion of BOXSHALL (2004); this discussion is an important theoretical base for manuscript 5 and manuscript 6.

Authors' contribution: DM designed the study, performed most of the experiments, interpreted the data, elaborated the theoretical part and drafted the manuscript; AM conceived the study, revised the intellectual content and helped to draft the manuscript; MR conceived and designed the study, performed some experiments and interpreted the data; GF revised the intellectual content and helped to draft the manuscript.

MANUSCRIPT 2

TITLE: Segmental pattern formation following amputation in the flagellum of the second antennae of *Asellus aquaticus* (Crustacea, Isopoda).

AUTHORS: Diego Maruzzo, Mia Egredzija, Alessandro Minelli and Giuseppe Fusco.

STATUS: in press in the *Italian Journal of Zoology*.

This manuscript describes in detail the production of new articles in the flagellum of the second antennae of *A. aquaticus* following amputation along flagellum. Most of the data on which the paper is based are the product of two master theses (MARUZZO, 2003; EGREDZIJA, 2006). This work completes the data of the previous manuscript, thus allowing a more complete understanding of the mechanism of article production during regeneration in the second antennae of *A. aquaticus*.

Authors' contribution: DM designed the study, performed experiments, interpreted the data, elaborated the theoretical part and drafted the manuscript; ME performed experiments and interpreted the data; AM and GF revised the intellectual content and helped to draft the manuscript.

MANUSCRIPT 3

TITLE: Comparative morphology and development of isopod (Crustacea) second antennae.

STATUS: unpublished manuscript.

This manuscript describes the morphology and the mechanism of new article production during postembryonic development of the second antennae of the isopods *Idotea chelipes*, *Lirceus fontinalis* and *Sphaeroma serratum*. Since observations were made with both light and scanning electron microscopy (SEM) while for *Asellus aquaticus*, which is the main term of comparative reference, SEM pictures were not yet available, original SEM observation on this species are also provided. This work completes the discussion in manuscript 1 providing original observations useful for understanding the evolution of both the antennal flagellum morphology and the mechanism of new article production during postembryonic development in isopod.

MANUSCRIPT 4

TITLE: Segmental mismatch in arthropod appendages: the naupliar antennal exopod of *Artemia* (Crustacea, Branchiopoda, Anostraca).
AUTHORS: Diego Maruzzo, Alessandro Minelli and Giuseppe Fusco.
STATUS: in advanced status of preparation for eventual submission to *Arthropod Structure & Development*.

This manuscript describes the external morphology and the muscular system of the naupliar second antennal exopod of *Artemia* sp. This work was meant to investigate the relationship between muscles, muscle insertions and joints; however, no complete joints are present in this model. These unexpected results gave the opportunity to review and discuss the occurrence of segmental mismatch in arthropod appendages. Special attention was also paid to the relationship between muscle insertions and cuticular folds.

Authors' contribution: DM designed the study, performed experiments, interpreted the data, elaborate the theoretical part and drafted the manuscript; AM and GF revised the intellectual content and helped to draft the manuscript.

MANUSCRIPT 5

TITLE: Article number and muscular system in the naupliar appendages.
STATUS: unpublished manuscript.

This manuscript provides a review on the ontogenetic variation in article number and on the structure of the muscular system in the naupliar appendages. The muscular system of the naupliar appendages of a cirripede (*Balanus improvisus*) is also described here for the first time. The data are then discussed in a comparative way to elucidate, following the discussion in the manuscript 1, the ontogenetic difference between true articles and annuli.

MANUSCRIPT 6

TITLE: On the anatomy of some articles in arthropod appendages.
STATUS: unpublished manuscript.

This manuscript described the musculature with specific attention to the muscular insertion pattern in the antennae of the centipede *Lithoubius forficatus* and in the pleopods of the amphipod *Gammarus roeselii*. The anatomical base of the distinction between true article and annuli is then discussed in the light of these data and of the conclusions of the previous manuscript.

MANUSCRIPT 7

TITLE: On the proximal "growth zone" of arthropod appendages.
STATUS: unpublished manuscript.

This manuscript described the mechanism of article production during postembryonic development in the pleopodal rami of the amphipod *Gammarus roeselii* as well as the development of the epidermis in the same model and in the second antennal flagellum of *Asellus aquaticus*. A specific proximal "growth zone" in arthropod appendages is defined and its occurrence is discussed.

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GROWTH AND REGENERATION OF THE SECOND ANTENNAE OF *ASELLUS AQUATICUS* (CRUSTACEA, ISOPODA) IN THE CONTEXT OF ARTHROPOD ANTENNAL SEGMENTATION[*]

ABSTRACT

The production of new articles in the flagellum of the second antennae of *Asellus aquaticus* was studied in both undamaged and regenerating antennae. Segmentation is an iterative process in two phases: a) the first proximal flagellomere (the meristematic article) repeatedly gives off distally a new primary article; b) each primary article divides into four secondary articles (a quartet). To a certain extent, production and development of different quartets are independent processes. Evidence is provided that the formation of new articles and their setae are partly decoupled. During regeneration from the preferred breakage point (the so-called "autotomy plane"), the flagellum is generated by the same mechanism of two-phase segmentation. The regenerated flagellum has a normal segmental composition, except for the tip (the apical complex), which has four flagellomeres rather than the normal five. The similar segmental pattern observed also in other malacostracan crustaceans and in insects, supports a close phylogenetic relationship among the two groups; if the latter proves not to hold, that similarity would provide an example of parallel evolution of developmental mechanism. The difference between "true" articles and annulations, defined on the structure of the muscular system, is discussed on the basis of comparative developmental data. In general, annulations are produced more sequentially, compared with the almost simultaneous emergence of true articles.

INTRODUCTION

The study of segmentation of arthropod appendages is an interesting chapter of evolutionary developmental biology (WILLIAMS & NAGY, 1996; WILLIAMS, 1999, 2004; ABZHANOV & KAUFMAN, 2000; MINELLI *et al*., 2000; BITSCH, 2001; SCHRAM & KOENEMANN, 2001). Nevertheless, very little attention has been paid to date to those arthropod appendages, like most antennae, where the number of articles increases during postembryonic development. Few reviews are available on this subject. IMMS (1940) summarised knowledge about the postembryonic development of hexapod antennae. More recently, BOXSHALL & HUYS (1998) proposed a general model of segmentation for the first antennae (antennules) of copepods.

Segmentation of this kind of appendages usually involves the production of new annulations and not of "true" articles (sensu BOXSHALL, 2004). The structural difference between these two segmental units has been often overlooked in the discussion about arthropod limb evolution (BOXSHALL, 2004), and few data for comparative developmental analysis are available.

The old descriptive literature, however, is full of insightful detail that deserves reappraisal from a modern viewpoint. For example, by comparing the morphology of several species of aselloid isopods, RACOVITZA (1925) hypothesized a general model of antennal segmentation, but also raised problems, mainly concerning the formation of new articles during regeneration. However,

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his model was not supported by experiments, and lacked longitudinal studies of individual ontogenies.

The second antennae of the freshwater isopod *Asellus aquaticus* (Linnaeus, 1758) are uniramous appendages composed of a proximal peduncle and a distal flagellum. The peduncle is composed of six articles with intrinsic muscles, and has a preferred breakage point (PBP), often called the autotomy plane, between the fourth and the fifth article. The flagellum is devoid of muscles (WEGE, 1911), and its articles (flagellomeres) increase in number during the whole life, as this species never stops moulting. Along the flagellum there are sensory setae of uniform shape, with characteristic distribution on a few, different positions on the distal part of each article (with few exceptions, see below). Each article bears one row of setae (WEGE, 1911; RACOVITZA, 1925), and the number and position that the setae can occupy are distinctive of specific articles along the flagellum.

Antennal regeneration of *A. aquaticus* is well-known (e.g. PRZIBRAM, 1899; ZUELZER, 1909; WEGE, 1911), but there is no study of segmentation during regeneration. In this work we studied the segmentation of the second antennae of *A. aquaticus* during normal development as well as during regeneration following breakage at the PBP. Regeneration following cuts at other positions along the flagellum will be treated in a forthcoming article.

MATERIALS AND METHODS

Specimens of *A. aquaticus* were collected in a stream in locality Polegge (Vicenza, northern Italy) from May 2002 to May 2003, and reared in the laboratory with water and plant debris from the same site. Observations and experiments were performed from May 2002 to June 2003.

Females with brood pouch were isolated and checked daily to collect juveniles. Juveniles released from the brood pouch were moved to individual Petri dishes and checked regularly to collect exuviae. The collected exuviae were mounted in ethylene glycol immediately or after storage in 70% ethanol. Fixed specimens were also studied. As needed, specimens were cleared in lactophenol, duration of treatment depending on size.

Slides were observed in bright field, phase contrast or Nomarski interference. Additional in vivo observations were performed by manipulating the specimens as described below for regeneration experiments.

The schedule of production of new articles was tracked by the help of morphological markers (setae). Faint article boundaries, often characteristic of articles in the course of division, also contributed to the assessment of the segmentation schedule. Observations on the postembryonic development of 88 specimens were performed. For every specimen several exuviae were collected, thus allowing to study individual ontogenies. Additional adult and subadult specimens were also observed.

Regeneration experiments were performed on young (0–60 days) specimens, as these moult and regenerate faster than later stages. Specimens were placed on a slide in a drop of water, the slide was then placed above a Petri filled with ice to reduce the specimen's activity. Cuts were performed under a dissection microscope using fine scissors or a microtome knife. Only one antenna was cut, usually the right one. After amputation the specimens were moved back to their Petri dishes and subsequent exuviae were collected and studied as described.

Regeneration experiments were performed on 33 specimens. For 19 specimens the antenna was cut at the PBP; for the remaining 14 the cut was also in the peduncle, but distal to the PBP. In the latter cases the stumps were subsequently reduced (by an undetermined mechanism: autotomy, autospasy or autotilly; see BLISS, 1960) at the PBP without any further intervention.

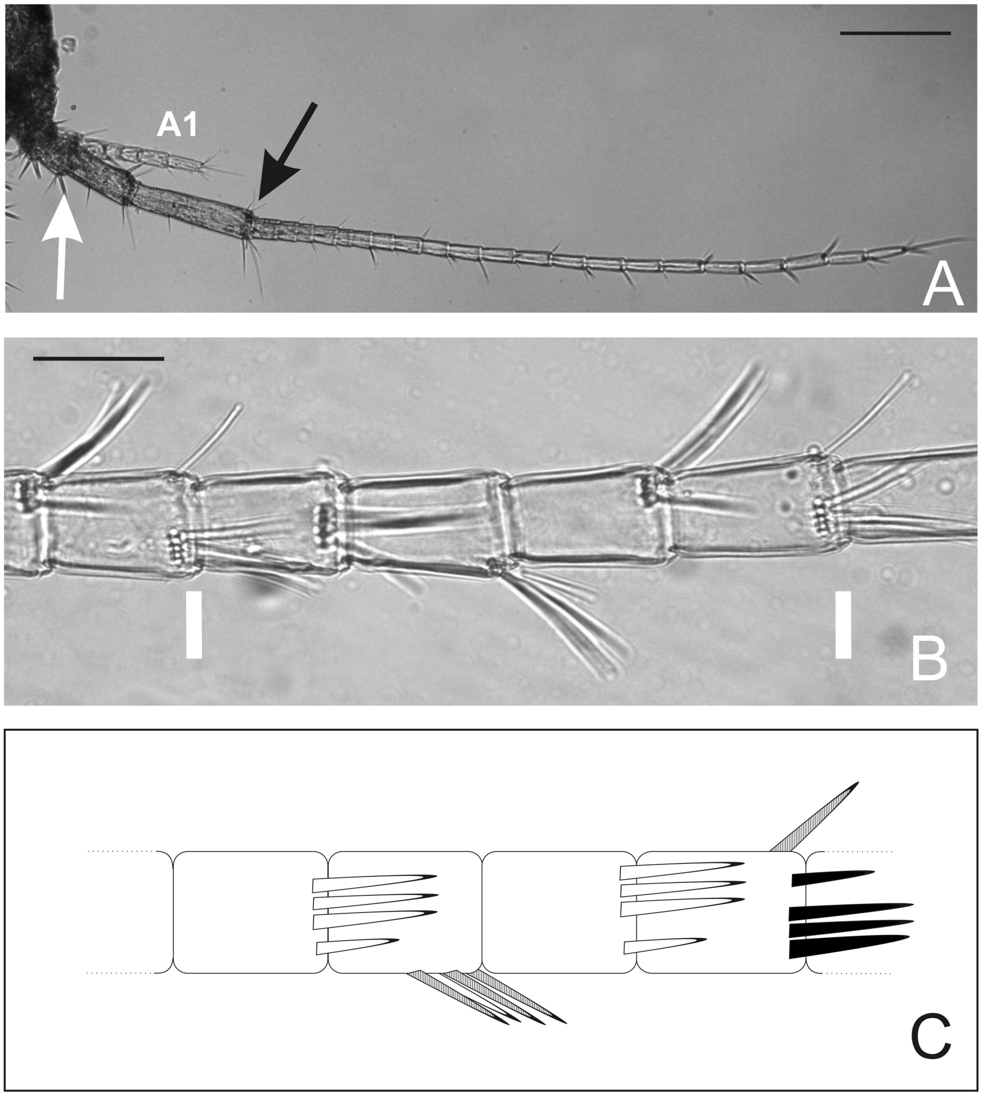

Fig. 1.1. Second antenna of *Asellus aquaticus*. A, dorsal view. White arrow points at the preferred breakage point (PBP), black arrow at the edge between peduncle and flagellum. A1, first antenna. Scale bar 200 µm. B, a complete quartet, bracketed within the white marks, of a left antenna. Scale bar 50 µm. C, schematic representation of a complete quartet. Dorsal setae are represented in black, lateral setae in grey, ventral setae in white.

RESULTS

STRUCTURE AND SEGMENTATION OF THE FLAGELLUM

The general morphology of the second antennae is shown in Fig. 1.1A.

Most of the flagellar length can be described as a series of sets of four articles having an individually specific setal pattern (number and position of setae), we call these units complete quartets (Fig. 1.1B, C). The whole flagellar portion composed of complete quartets is called central region. A different sequence of flagellomeres is found in the proximal meristematic region and in the distal region of the flagellum (apical complex) (for a detailed morphological description of the whole flagellum, see RACOVITZA, 1925).

The meristematic region is where new quartets are formed. The production of new quartets follows a stereotyped scheme of segmentation as first hypothesized by RACOVITZA (1925) on the basis of the morphology of the flagellum. Segmentation proceeds in two phases. First, the most proximal flagellar article (the meristematic article) divides into two articles: a proximal copy of itself and a distal article. This distal article is the founder of a future quartet (one-article incomplete quartet; Fig. 1.2). Second, the one-article incomplete quartet produces a complete quartet by growing and dividing in successive steps, thus passing through the conditions of two- and three-article incomplete quartet. This pattern of division follows a precise sequence (Fig. 1.3): the one-article incomplete quartet divides in two articles, of which the distal one divides before the proximal one. The meristematic region is composed of the meristematic article, followed by a variable number of incomplete quartets arranged in a proximo-distal gradient of "maturity", those closer to the state of complete quartet being always distal to the less complete ones (Fig. 1.4).

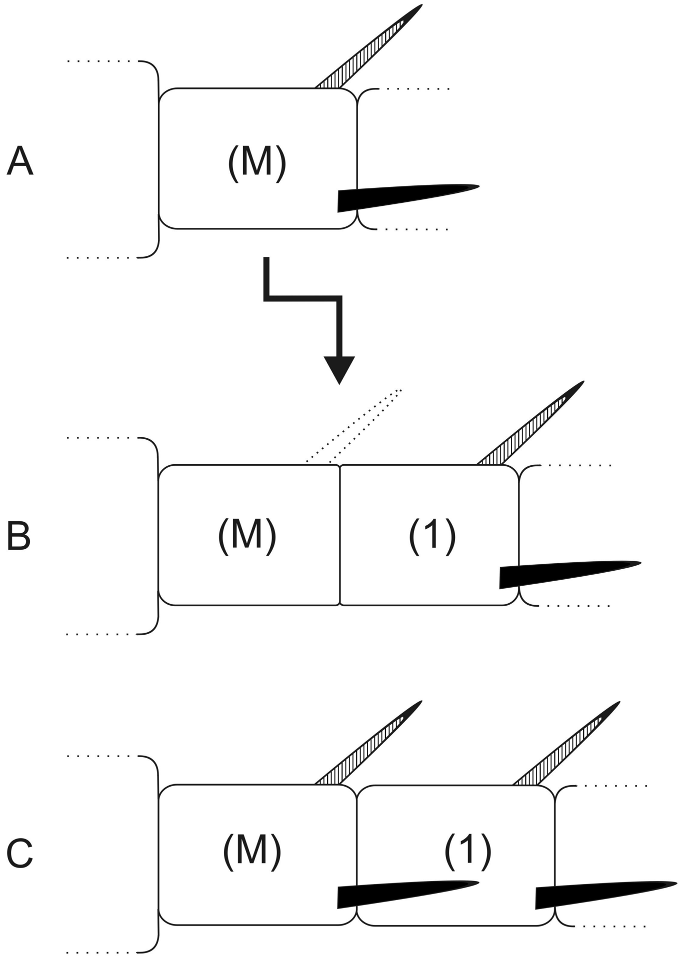

Fig. 1.2. Schematic representation of the division of the meristematic article in the flagellum of the second antennae of *Asellus aquaticus* (left antenna seen from above, distal end to the right). Orientation and symbols as in Fig. 1.1C, setae whose first appearance does not necessarily coincide with the first appearance of the next distal article boundary are dashed. Arrow points to the new article boundary. (M), meristematic article; (1), one-article incomplete quartet. The meristematic article can be unarmed or bear one or two seta(e), depending on its developmental status; these setae will be "inherited" by the one-article incomplete quartet that will form after the division. The A to B to C sequence may be accomplished in one to three moults.

The meristematic region is followed by a variable number of complete quartets forming the central region. The number of complete quartets increases during ontogeny.

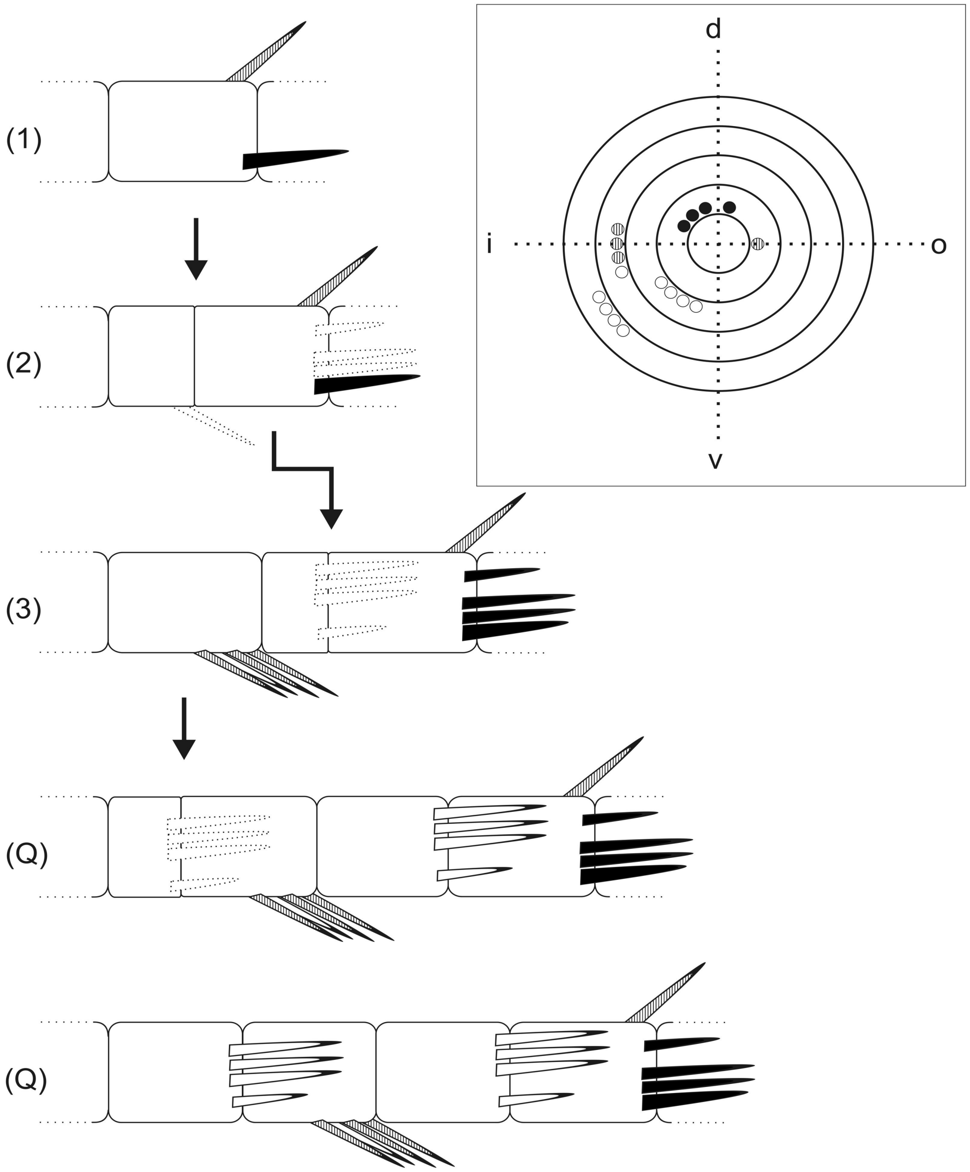

Fig. 1.3. Schematic representation of the stereotyped division pattern of one-article incomplete quartet. (1), one-article incomplete quartet; (2), two-article incomplete quartet; (3), three-article incomplete quartet; (Q), complete quartets, here shown with two different degrees of development of article size and setae. Orientation and symbols as in Figs. 1-2. In the frame, an apical view of a complete quartet; locations of the setae around the articles is indicated by the circles, black, grey, or white according to the coding in Figs. 1.1-1.2. d, dorsal side; v, ventral side; i, inner side; o, outer side.

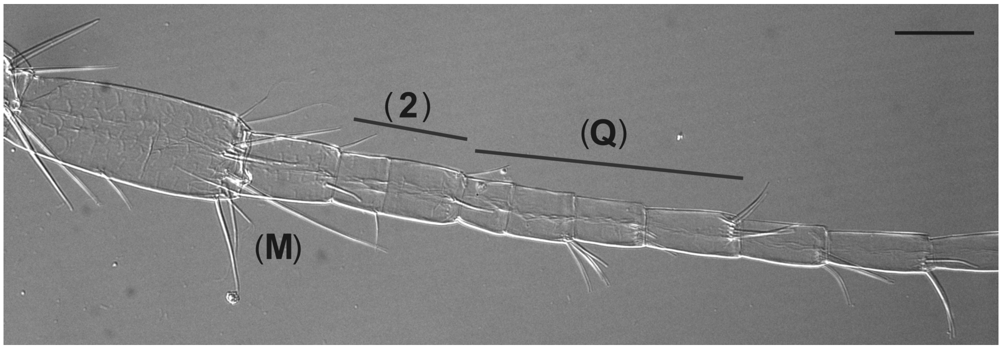

Fig. 1.4. An example of meristematic region and proximal part of the central region of a second antenna flagellum of *Asellus aquaticus*. (M), meristematic article; (2), two-article incomplete quartet; (Q), complete quartet. Scale bar 50 µm.

The apical complex is composed of the distal article (apex) which is cone-shaped and differs in the setal pattern from all other articles in the flagellum. The apex is preceded by four articles whose setal pattern does not match with that of a quartet. Undamaged antennae thus have an apical complex of 5 articles, with a specific setal pattern (Fig. 1.5). The three proximal articles of this apical complex have the same setal pattern as the first, second and fourth article of a complete quartet (see Fig. 1.1B-C). The penultimate article has a setal pattern intermediate between those of the first and the third article of a quartet.

MORPHOGENESIS DURING NORMAL DEVELOPMENT

At hatching, the second antennae of *A. aquaticus* are composed more frequently of 12 flagellomeres, but newly hatched specimens with 11 or 10 flagellomeres are common (27% and 18% of the observed cases, respectively). From proximal to distal, a 12-flagellomere flagellum has the following composition: the meristematic article, one two-article incomplete quartet, one complete quartet, and the apical complex.

The postembryonic development of the flagellum then involves both the production of new one-article incomplete quartets by the meristematic article, and their division to produce new complete quartets. Once a new one-article incomplete quartet has been produced, its segmentation proceeds autonomously, invariably following the stereotyped pattern just described. However, there must be some form of control over the schedule of production and division of the several incomplete quartets at the level of the entire meristematic region, because there are limits to the range of the number of incomplete quartets and because there is a proximo-distal gradient of maturity among incomplete quartets. This limited set of segmental patterns suggests the existence of developmental constraints on the relative timing of segmentation among different incomplete quartets.

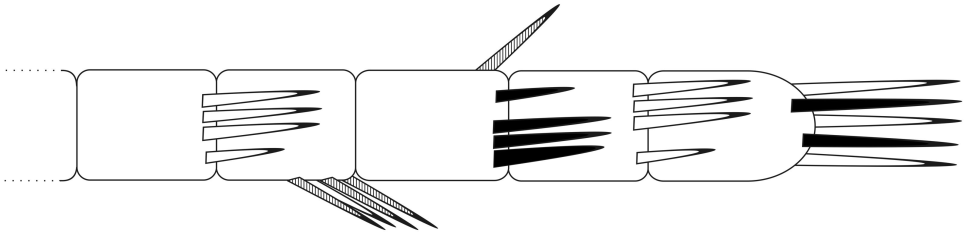

Fig. 1.5. Schematic representation of the normal apical complex (orientation and symbols as in Figs. 1.1-1.2).

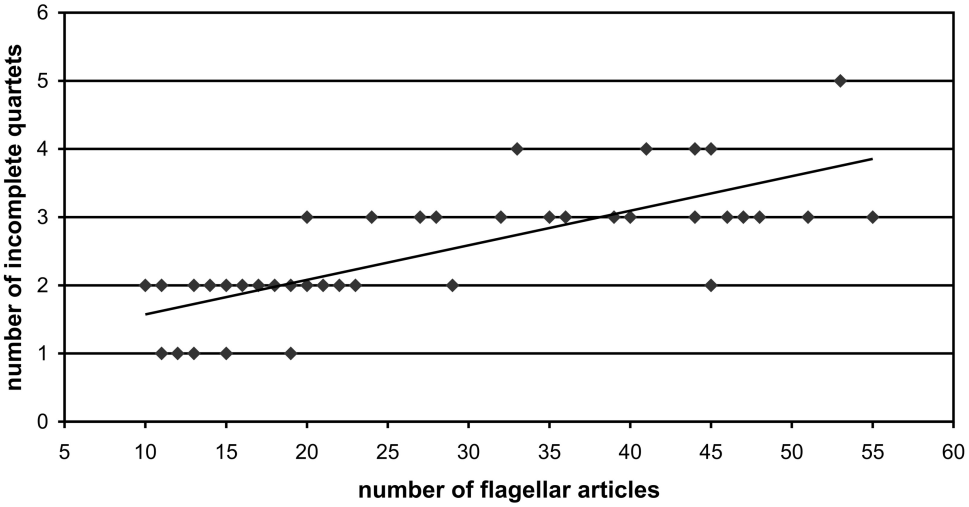

Fig. 1.6. Relationship between the number of incomplete quartets and the number of flagellar articles in undamaged flagella. Regression line is shown.

In early postembryonic stages, only one or two incomplete quartets are present. In later stages, as the number of flagellomeres increases, the mean number of incomplete quartets also increases (linear regression, two-tailed t-test, $p<0.001$, $n=40$, Fig. 1.6). In flagella with a short meristematic region, the proximo-distal gradient of maturity is tight, so there is no repetition of equally developed incomplete quartets, e.g., two one-article incomplete quartets are not found. As the flagellum gets longer, the number of incomplete quartets increases and some repetition of equally developed incomplete quartets occurs. Two consecutive one-article, two-article, and three-article incomplete quartets were found in flagella with at least 41, 27, and 53 flagellomeres respectively.

The detailed segmentation schedule of the flagellum varies among specimens and, to a lesser extent, even between the two flagella of the same specimen. This variation in time produces a variation in segmental arrangement, so that flagella with the same number of articles can actually exhibit different quartet composition. During normal development from 11 to 16 flagellomeres, flagella with 11, 13 or 15 articles are found with either of two alternative quartet compositions, whereas flagella with 12, 14 or 16 articles present only one. This pattern can be easily explained as a by-product of the constraints on segmentation timing outlined above (Fig. 1.7).

The frequency at which different quartet compositions are found is different. This is because some quartet compositions may persist for more than one moult cycle, while others are both produced more rarely and do not last for long (Table 1.1). During the early postembryonic development the number of articles added at each moult follows some regularities. About 50% of the antennae with an even number of flagellomeres do not increase article number at the next moult, while about 30% acquire two flagellomeres. No regularity is found in longer antennae.

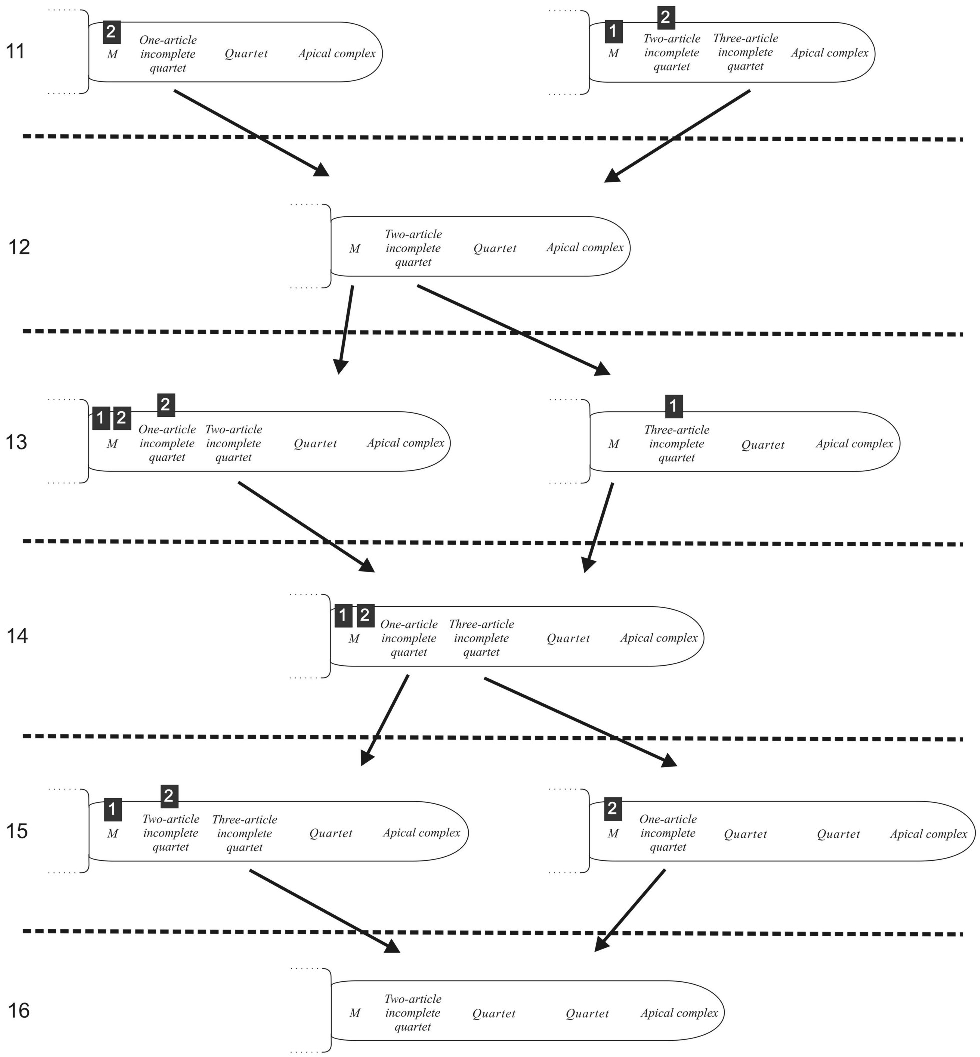

Fig. 1.7. Schematic representation of the changes in segmental composition in the flagellum during normal development from 11 to 16 flagellomeres (numbers on the left). Black tags indicate the meristematic article and the incomplete quartets that do not divide because: [1] divisions would produce a too short or a too long meristematic region (outside the observed range); [2] divisions would produce two consecutive incomplete quartets with the same number of articles, defeating a tight gradient of maturity.

Table 1.1. Individual flagella in subsequent developmental stages of early postembryonic development. Pre-moult segmental composition and the corresponding post-moult status are shown; the total number of flagellomeres is given in square brackets. Segmental composition is summarised as follow: (M), meristematic article; (1), one-article incomplete quartet; (2), two-article incomplete quartet; (3), three-article incomplete quartet; (Q), complete quartet; (AC), apical complex. Percentages have been calculated only where at least 10 cases were available.

Pre-moult segmental composition		Post-moult segmental composition		N. of observed cases	%
[11]	(M) (1) (Q) (AC)	[12]	(M) (2) (Q) (AC)	2	-
N = 2					-
[11]	(M) (2) (3) (AC)	[12]	(M) (2) (Q) (AC)	3	-
N = 7		[13]	(M) (3) (Q) (AC)	3	-
		[14]	(M) (1) (3) (Q) (AC)	1	-
[12]	(M) (2) (Q) (AC)	[12]	(M) (2) (Q) (AC)	13	52.0 %
N = 25		[13]	(M) (3) (Q) (AC)	4	16.0 %
		[13]	(M) (1) (2) (Q) (AC)	1	4.0 %
		[14]	(M) (1) (3) (Q) (AC)	7	28.0 %
[13]	(M) (3) (Q) (AC)	[13]	(M) (3) (Q) (AC)	2	-
N = 3		[14]	(M) (1) (3) (Q) (AC)	1	-
[13]	(M) (1) (2) (Q) (AC)	[14]	(M) (1) (3) (Q) (AC)	10	100.0 %
N = 10					
[14]	(M) (1) (3) (Q) (AC)	[14]	(M) (1) (3) (Q) (AC)	17	50.0 %
N = 34		[15]	(M) (1) (Q) (Q) (AC)	2	5.9 %
		[15]	(M) (2) (3) (Q) (AC)	2	5.9 %
		[16]	(M) (2) (Q) (Q) (AC)	12	35.3 %
		[18]	(M) (1) (3) (Q) (Q) (AC)	1	2.9 %
[15]	(M) (1) (Q) (Q) (AC)	[16]	(M) (2) (Q) (Q) (AC)	1	-
N = 1					
[15]	(M) (2) (3) (Q) (AC)	[15]	(M) (2) (3) (Q) (AC)	2	-
N = 6		[16]	(M) (2) (Q) (Q) (AC)	3	-
		[17]	(M) (1) (2) (Q) (Q) (AC)	1	-
[16]	(M) (2) (Q) (Q) (AC)	[16]	(M) (2) (Q) (Q) (AC)	12	63.2 %
N = 19		[17]	(M) (1) (2) (Q) (Q) (AC)	1	5.3 %
		[18]	(M) (1) (3) (Q) (Q) (AC)	6	31.6 %
[17]	(M) (1) (2) (Q) (Q) (AC)	[17]	(M) (1) (2) (Q) (Q) (AC)	2	16.7 %
N = 12		[18]	(M) (1) (3) (Q) (Q) (AC)	6	50.0 %
		[19]	(M) (2) (3) (Q) (Q) (AC)	4	33.3 %
[18]	(M) (1) (3) (Q) (Q) (AC)	[18]	(M) (1) (3) (Q) (Q) (AC)	9	47.4 %
N = 19		[19]	(M) (2) (3) (Q) (Q) (AC)	5	26.3 %
		[20]	(M) (2) (Q) (Q) (Q) (AC)	3	15.8 %
		[21]	(M) (1) (2) (Q) (Q) (Q) (AC)	2	10.5 %
[19]	(M) (2) (3) (Q) (Q) (AC)	[19]	(M) (2) (3) (Q) (Q) (AC)	2	14.3 %
N = 14		[20]	(M) (2) (Q) (Q) (Q) (AC)	2	14.3 %
		[20]	(M) (1) (2) (3) (Q) (Q) (AC)	3	21.4 %
		[21]	(M) (1) (2) (Q) (Q) (Q) (AC)	6	42.9 %
		[22]	(M) (1) (3) (Q) (Q) (Q) (AC)	1	7.1 %
[20]	(M) (2) (Q) (Q) (Q) (AC)	[21]	(M) (1) (2) (Q) (Q) (Q) (AC)	1	-
N = 2		[22]	(M) (1) (3) (Q) (Q) (Q) (AC)	1	-
[21]	(M) (1) (2) (Q) (Q) (Q) (AC)	[22]	(M) (1) (3) (Q) (Q) (Q) (AC)	4	-
N = 6		[23]	(M) (2) (3) (Q) (Q) (Q) (AC)	2	-

MORPHOGENESIS DURING REGENERATION

To cut the antenna at the PBP means to remove the two distalmost articles of the peduncle and the entire flagellum (Fig. 1.1A). When the regenerate first appears (at the first or at the second post-operative moult) it is already composed of the two distalmost articles of the peduncle, plus between 4 and 15 new flagellomeres.

In comparing segmentation schedules of undamaged and regenerating antennae, regenerating antennae with N flagellomeres must be compared with undamaged antennae with N-1 flagellomeres, because a flagellum regenerated from the PBP always has an apical complex of four articles instead of the normal five. The production of this regenerated apical complex follows very closely the stereotyped division sequence of a complete quartet during normal development, except for the last article which assumes the morphology of the apex (Fig. 1.8).

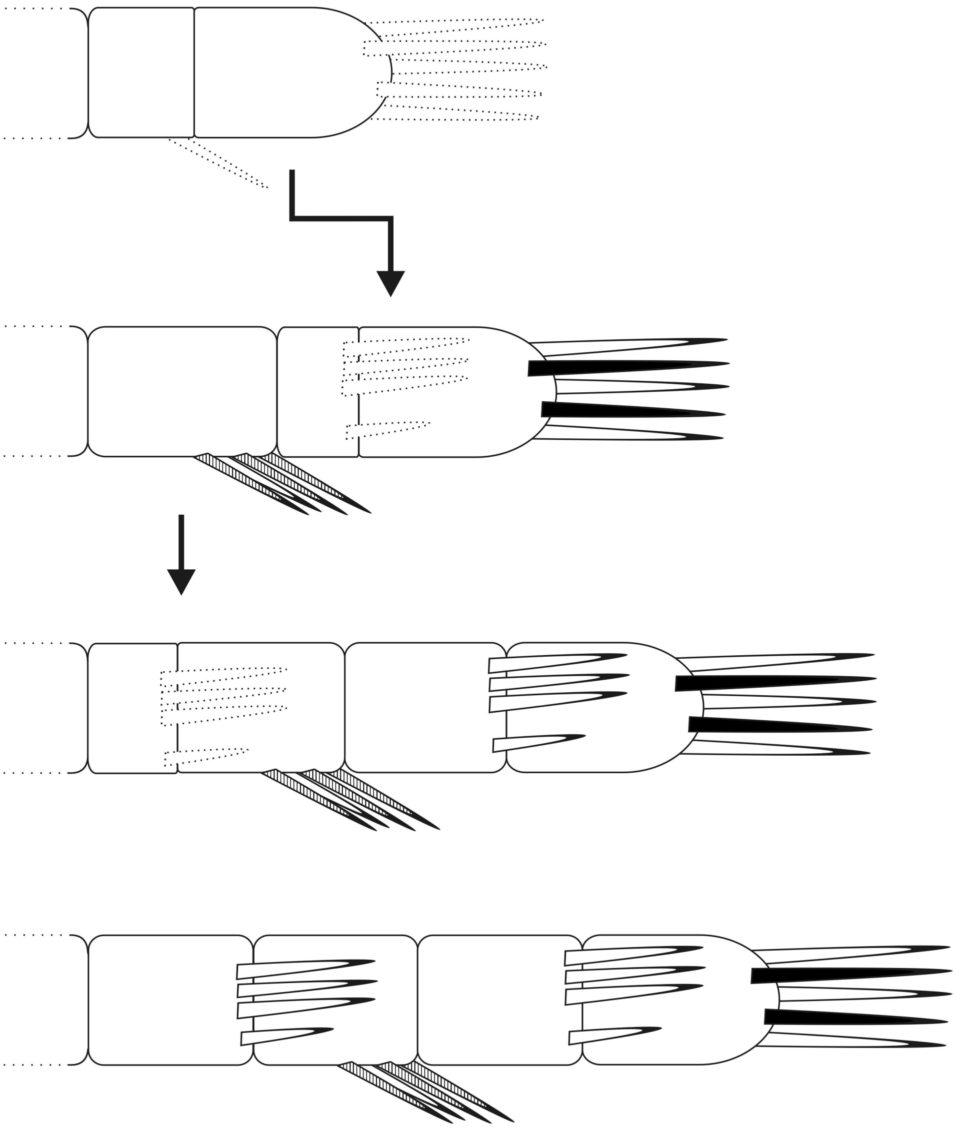

Fig. 1.8. Schematic representation of the genesis of the apical complex following regeneration from the PBP. Number and location of the setae in the apex show intraspecific variability (orientation and symbols as in Figs. 1.1-1.2).

In the meristematic region new quartets are produced by the same mechanism observed in normal development. However, the segmentation scheduling is more variable (Fig. 1.9). Comparing regenerating antennae with N articles to the corresponding undamaged ones with N-1 articles, during regeneration the meristematic region may include a larger number of incomplete quartets (compare for instance the antennae with 11 flagellomeres in Fig. 1.7 with the ones with 10 in Fig. 1.9, and the antenna with 16 flagellomeres in Fig. 1.7 with the one with 15 in Fig. 1.9). Also, incomplete quartets with the same article number are found in the same flagellum earlier in the developmental schedule, i.e., in shorter flagella (see antenna with 10 flagellomeres in Fig. 1.9). This is reflected in the fact that, in most cases, a given number of flagellar articles may correspond to two different quartet compositions.

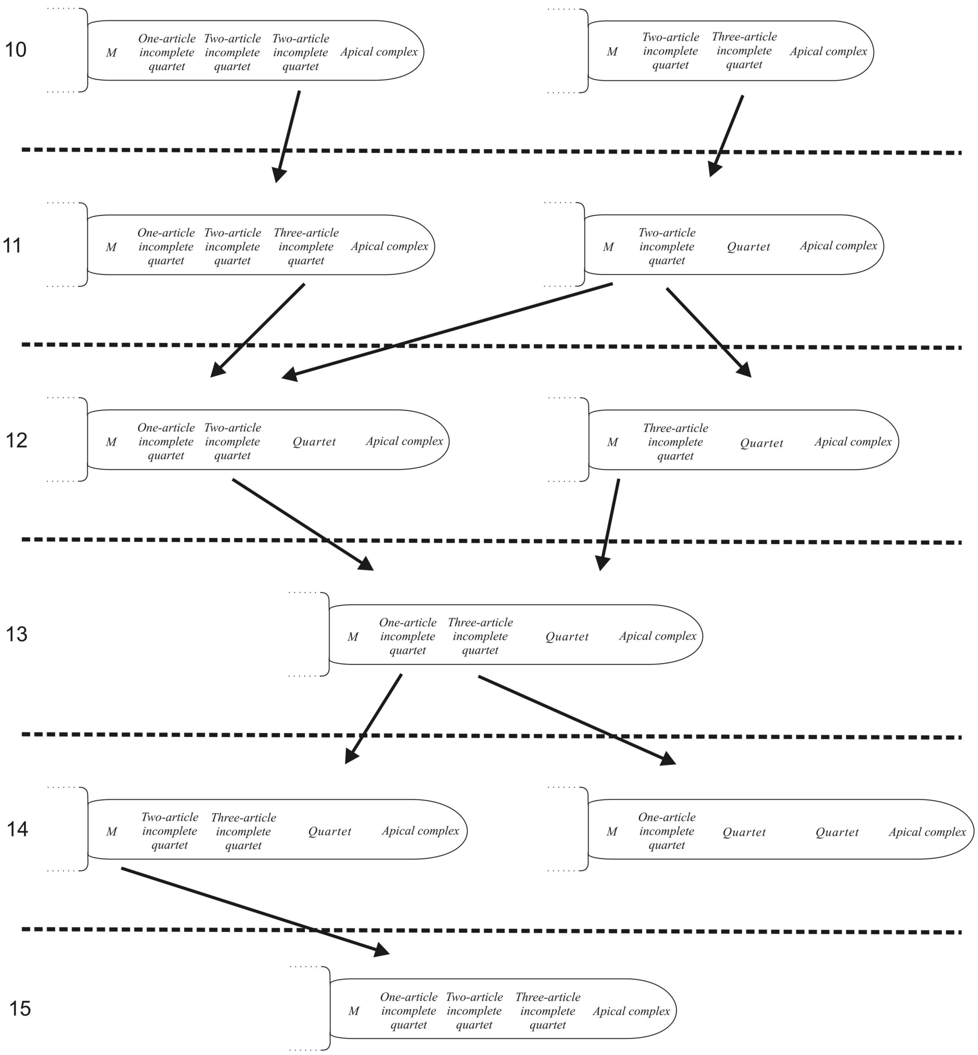

Fig. 1.9. Schematic representation of the changes in segmental composition of the flagellum during regeneration from the PBP from 11 to 16 flagellomeres. The apical complex is hypomorphic, with only four articles (see text). Numbers on the left are the total number of flagellomeres.

FORMATION OF ARTICLES AND FORMATION OF SETAE

The time of formation of new articles does not correlate strictly with the time of formation of their setae. The meristematic article (or an article of an incomplete quartet) can divide when it has already one or more setae at mid-length, or not.

For the dividing articles of incomplete quartets these mid-length setae will be "inherited" by the proximal one of the two "offspring" articles. It is not rare, anyway, to find unarmed articles, i.e. articles that will develop their setae only following one more moult.

RACOVITZA (1925) recorded "incomplete quartets" with four rows of setae in a sequence comparable to that of a normal complete quartet. This implies some articles having more than one row of setae. Racovitza interpreted these cases as the product of a regeneration process. Three comparable cases were observed in our material (regenerated antennae only). These articles are the product of a normal setal development associated to a failure of the article boundary formation (Fig. 1.10).

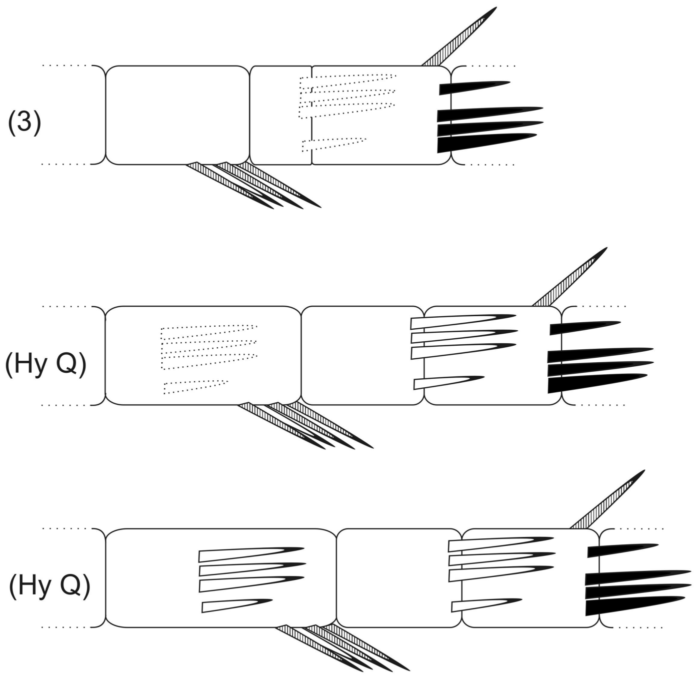

Fig. 1.10. Schematic representation of one of the rarely occurring articles with a double row of setae. In this example a three-article incomplete quartet (3) produces a hypomorphic quartet (of three articles only (Hy Q) here shown with two different degrees of development of article size and setae) with the setal pattern typical of a complete quartet (orientation and symbols as in Figs. 1.1-1.2).

DISCUSSION

THE DEVELOPMENT OF THE FLAGELLUM

The process of segmentation of the flagellum of the second antennae in *A. aquaticus* is both iterative, as the two-phase process is repeated several times, and intercalary, since new elements are inserted between older ones. Primary articles are produced by the meristematic article, and these are subsequently divided into a fixed number of secondary articles.

Contrasting with the production of primary articles, which is a sequential, virtually open process, the subsequent subdivisions of each primary article into four final segmental units is closed and stereotyped, the distalmost article of a two-article incomplete quartet being always the first to divide.

A proximo-distal gradient of maturity is present along the series of incomplete quartets. When the number of incomplete quartets is small enough (short meristematic region), the gradient is tight, not allowing the repetition of equally developed incomplete quartets. The number of incomplete quartets is correlated with size and/or segmentation of the whole antenna, both in normal development and regeneration.

The main exception to this segmentation process is the apical complex. Paradoxically enough, there are more problems in trying to explain the formation of the normal apical complex than the regenerated one, as it is the former that does not follow the usual segmentation path of the flagellum.

COMPARATIVE DEVELOPMENT OF ISOPOD ANTENNAE

The segmentation model illustrated here for the second antennae of *A. aquaticus* can be possibly applied to most of other aselloid isopods, because their second antennae are patterned in quartets. RACOVITZA (1925) already provided examples for the Asellidae (several species of *Asellus* and *Caecidotea stygia* Packard, 1871 (sub *Caecidothea stygia*)) and the Stenasellidae (*Protelsonia gjorgjevici* (Racovitza, 1924) and *Stenasellus virei* Dollfus, 1897). However, among the aselloids studied by RACOVITZA (1925), there were also species expressing a systemic deviation from this model. The central part of the flagellum of the second antennae of *Mancasellus* (now *Lirceus*) is composed of units of two articles, rather than four. However, these two-article units bear the regular aselloid quartet configuration of four rows of setae, as each article of the central region of the antenna bears two rows of setae, one at mid-length of the article and one on its distal part.

Among valviferan isopods, new articles of the second antenna flagellum are produced by the division of the first flagellomere only. This was reported for several species of *Idotea* (NAYLOR, 1955) and for *Synisoma nadejda* Rezig, 1989 (EL HEDFI-BEL HAJ KHELIL, 2002; and references therein).

The flagellum of the first antennae of the asellids develops in a way similar to that of the second antennae. New primary articles are produced by the fifth antennomere. In *A. aquaticus* these primary articles divide only once, producing two secondary articles (M. RONCO, unpublished data), while in *Lirceus* they remain undivided (ZELENY, 1907). Thus, the 2:1 ratio between quartets in *Asellus* second antennae and two-article units in *Asellus* first antennae mirrors the 2:1 ratio between two-article units in *Lirceus* second and one-article units in *Lirceus* first antennae. On the basis of morphological observations of the first antennae in many isopods, among which was *A. aquaticus*, WÄGELE (1983) considered the fifth antennal article (the article where we see meristematic activity) as the second flagellomere. This would imply that the meristematic article is not the first flagellomere. We do not have original data on that, but, on the basis of anatomical observations (muscles and tegument), VON KAULBERSZ (1913) long proposed to consider the fifth antennal article of the first antennae of *A. aquaticus* as the first flagellomere.

Since the condition here described for *A. aquaticus* is found in the second antennae of other species of Asellidae as well as Stenasellidae (see above), we can infer that this is the plesiomorphic condition for the Asellidae. The different condition found in *Lirceus* (Asellidae) is thus a derived one. Due to the limited taxonomic sampling on isopod antennal development, it is not possible to infer a plesiomorphic developmental condition for isopods as a whole. There is, however, variation (see above). It is interesting to note that all kinds of evolutionary variation in the antennal flagellum segmentation process reported above deal only with the second phase of the process, i.e., with the subsequent divisions of articles produced by the meristematic one.

COMPARATIVE STRUCTURE AND DEVELOPMENT OF ARTHROPOD ANTENNAE

Although there is currently no consensus about the phylogenetic relationships of the major arthropod groups, according to some phylogenetic hypothesis, malacostracan crustaceans and insects are phylogenetically closely related groups (see WILSON *et al.*, 2000; HARZSCH, 2002; FANENBRUCK *et al.*, 2004). The antennal flagellum of both these groups is characterized by being completely devoid of muscles (IMMS, 1939). This kind of structure is what BOXSHALL (2004) named "terminal annulation". Terminal annulations are not usually found in the antennae of other arthropods (IMMS, 1939). Exception are some springtails, e.g., *Sminthurus viridis* (Linnaeus, 1758) (IMMS, 1939; see also BOXSHALL, 2004), the first antennae of the anostracan *Caenestheriella australis* (Loven, 1847) (see BOXSHALL, 2004), and the first antennae of the ostracod *Uncinocythere occidentalis* (Kozloff and Whitman, 1954) (see SMITH & TSUKAGOSHI, 2005).

It seems reasonable to assume that terminal annulations in the antennae (both first and second) of malacostracans is an apomorphy of this group, or of a clade of malacostracans plus insects, if the latter will turn out to be their sister group. If the first and second antennae of malacostracans are homologous to the first and second antennae of "entomostracans", we can conclude that terminal annulations in the antennae emerged at the same time in both first and second antennae in malacostracans. This means that first and second antennae did not evolve independently, a supposition also supported by the similarities in the segmentation process of the flagellum in these two pairs of appendages (see above for isopods, below for other arthropods).

Few data are available for the segmentation of antennal flagella in other malacostracan crustaceans. The number of flagellar articles in the first antennae of the amphipod *Gammarus chevreuxi* Sexton, 1913 increases by division of the first and, sometimes, also of the second flagellomere (Sexton, 1924; quoted in SUTCLIFFE & CARRICK, 1981). In the lateral flagellum of the first antennae of the decapods *Cherax destructor* Clark, 1936 and *Panulirus argus* (Latreille, 1804) new articles are always produced at the base of the flagellum (SANDEMAN & SANDEMAN, 1996; STEULLET *et al.*, 2000), and in both species divisions in articles other than the first one are evident.

In hemimetabolous insects the first flagellomere was long recognised as the main producer of new articles (IMMS, 1940), and HENSON (1947) actually named it "meriston" for this reason. In the antennae of cockroaches (QUADRI, 1938; HAAS, 1955; CAMPBELL & PRIESTLEY, 1970; SCHAFER, 1973) and termites (FULLER, 1920) the primary articles divide once, producing two secondary articles each. In CAMPBELL & PRIESTLEY's (1970) nomenclature the articles produced by the “meriston” are called “meristal annuli” and the two articles produced by the division of one meristal annulus are called a “doublet”.

Similar developmental processes are thus found in the antennal flagellum of malacostracan crustaceans and insects. There is always a particular article (usually the first flagellomere) that is a specific “center of production” of new articles. However, as far as we are aware, regularity in secondary divisions has been found only in Asellota and in Dictyoptera. Diversity among the different crustacean and insect lineages may thus depend on the degree and regularity of secondary divisions. In the antennae of both “entomostracan” crustaceans and myriapods, no such developmental similarities can be found (e.g., BOXSHALL & HUYS, 1998 for copepods; SMITH & TSUKAGOSHI, 2005 for ostracods; MINELLI *et al*., 2000 for centipedes). In addition, as noted above, if we take into account the structure of the muscular system in the antennae, “entomostracan” crustaceans and myriapods clearly do not align with malacostracans and insects. If these similarities in flagellar structure and development in the antennae of malacostracans and insects are not due to the close phylogenetic relationship among the two groups, this would be an example of parallel evolution of a largely similar developmental mechanism for the segmentation of the flagellar structure with termial annulations.

DEVELOPMENT OF ANNULATIONS VERSUS “TRUE” ARTICLES

BOXSHALL (2004) clarified the difference between true articles and annulations in arthropod appendages. Annulations can be of two different kinds: terminal, if they are completely devoid of muscles (as are the antennal flagella studied in the present work), or intercalary, if they have a muscle or a tendon that pass through the annulations but does not insert on them. Boxshall also noted that annulations tend to appear later in the development of a limb if compared to true articles.

Furthermore, during postembryonic development, new appendages (or parts of appendages) composed of true articles are usually described as becoming completely segmented within a short period of time (e.g. during metamorphosis, in the case of the legs of a fly), after which they do not segment further. In these appendages, the formation of article boundaries is almost simultaneous. On the contrary, parts of appendages with annulations may, in general, increase article number over a longer period of time, across several moults: that is, the production of new annulations is more distinctly sequential than the production of true articles.

Insect legs are produced during embryogenesis or during metamorphosis, the last articles to be produced being those of the tarsus (BITSCH, 2001; KOJIMA, 2004): these articles are intercalary annulations. Furthermore, in some species, e.g., in the hemipteran *Oncopeltus fasciatus* (Dallas, 1852), the final tarsal segmentation is acquired during postembryonic development (SHAW & BRYANT, 1974). In crabs and lobsters, appendages that are formed during postembryonic development appear with their definitive segmental pattern, but for the annulated part of a limb, e.g., the exopod of the pereiopods in spiny lobster’s phyllosoma (DUGGAN & MCKINNON, 2003). A similar condition was reported also to occur during the regeneration of arthropod appendages. Here, true articles are all usually formed already when the regenerate first appears, while annulations increase in number over several moults (MARUZZO *et al.*, 2005).

In the present study we analysed a terminal annulated structure with indeterminate article number. This structure actually never stops adding new articles as long as there are moults available. A comparable behaviour has never been reported for parts of appendages composed of true articles.

However, there are appendages composed of true articles that increase in article number over sever moults. The first antennae of copepods do not have annulations, and new articles are produced during all the larval moults (BOXSHALL & HUYS, 1998). The musculature of this limb is rather simple, being composed of few muscles running from the base of the limb to the tip, and inserting in each article in between (BOXSHALL, 1985). New, intermediate, muscle insertions are assumed to be produced when new article boundaries are formed (BOXSHALL, 2004). In lithobiomorph centipedes, new antennal articles are produced during postembryonic development (LEWIS, 1981) and, again, their musculature is rather simple, with few muscles running from the base to the tip and inserting in each article in between (IMMS, 1939). In copepods, thoracopods are produced during the copepodid phase. They usually appear first as limb buds and later as incompletely segmented limbs. All the few subsequent additions of articles, anyway, involve only the rami (FERRARI, 1988). Thoracopod rami have also few muscles running from the base to the tip and inserting in each article in between (BOXSHALL, 1982, 1985).

So, despite the limited data available at present, we can conclude that the anatomical differences between true articles and annulations are matched by major differences in their development. The structure of the muscular system in a limb, in fact, correlates very well with its development. True articles are produced earlier in development and, usually, “all at once”, while annulations are produced later and in an “anamorphic-like” fashion. An intermediate condition is found in those (parts of) appendages where few muscles run along the whole length and insert in each article.

FORMATION OF ARTICLES VERSUS SETAE

In the present study, we used the setae as markers to track the formation of new articles, a method that has been widely used in developmental studies based on morphological techniques. However, even if the correlation between the formation of articles and the formation of setae is close enough for this purpose, the timing of production of setae and of articles of the second antennae of *A. aquaticus* are not as closely correlated. A newly formed article can be unarmed or bear a few setae. Also, following regeneration, an undivided article with a double rows of setae is sometimes formed. This is equivalent to two normal consecutive articles without a segmental boundary in between.

It should be noted that during the embryogenesis of *A. aquaticus* the antennal setae are formed simultaneously, just before hatching, when the flagellum has 10-12 flagellomeres (C. BIFFIS, personal communication). A similar event has been also recorded for the first antennae of *Lirceus* (ZELENY, 1907).

In different species of copepods, different correlations between homologous article boundaries and homologous setae have been described

(FERRARI & BENFORADO, 1998; FERRARI & IVANENKO, 2001). Also within the aselloids the correspondence between articles and rows of setae can be different: in most aselloids the structure is as described here, but there are exceptions, as *Lirceus*.

It seems safe to conclude that the positional information for the epidermal cells that will produce article boundaries and for those that will produce the setae is at most only partially derived by reciprocal interactions among these cells.

REGENERATION OF THE ANTENNAE: SIMILARITIES AND DIFFERENCES WITH RESPECT TO NORMAL DEVELOPMENT

In those few arthropods where antennal segmentation has been observed during both normal development and regeneration, it was noted that the two processes proceed in a similar way, e.g. in isopods (ZELENY, 1907; present study), in diplurans (IMMS, 1940), in cockroaches (SCHAFER, 1973), in centipedes (LEWIS, 2000), and in lobsters (STEULLET *et al.*, 2000; HARRISON *et a*l., 2003).

In the regeneration of *A. aquaticus* second antennae, after the formation of the blastema and regeneration of the distalmost articles, segmentation of the flagellum proceeds in a way very similar to normal development. New articles are acquired by the same mechanism of two-phase segmentation. However, due to an obvious acceleration of the morphogenetic process during regeneration (see SCHAFER, 1973 and HARRISON *et al.*, 2003 for a comparable increased activity of the "meristematic" region in cockroach and lobster respectively), the relationship between the number of flagellomeres and the number of incomplete quartets differs from that observed during normal development. Meristematic regions are comparably longer during regeneration with respect to undamaged antennae with the same numbers of articles, and it is rare to find a unique quartet composition for a given number of flagellomeres. Thus, even in short antennae, the gradient of maturity of the quartets is not tight, but allows for the repetition of equally developed incomplete quartets.

In the regeneration of the second antennae of *A. aquaticus* from the PBP, a specific "new" phenotype of the apical complex, with one flagellomere less than the normal one, was always obtained. In arthropods, regeneration abnormalities in the segmentation of the tegument, possibly produced by similar developmental mechanisms, are common. The hypomorphic regeneration very often observed in the insect tarsi (reviewed in MARUZZO *et al.*, 2005) is one of these. In cockroaches, regeneration from the PBP produces a tarsus with four articles instead of the normal five, the normal number is restored only if the amputation involves just one or two distalmost tarsal article(s) (TANAKA *et al.*, 1992).

CONCLUSION

Excluding a few distalmost articles, the flagellum of the aselloid second antennae develops by iterating a two-phase segmentation process: a meristematic article produces new "units" that grow and divide largely independently. This mechanism can also be hypothesized, with differences in the degree and regularity of secondary segmentation, for the segmentation of the flagella in other malacostracan crustaceans and in insects. In *Asellus*, following regeneration from the preferred breakage point, all articles are produced by the same two-phase mechanism. Since the distalmost articles are also produced by this mechanism, the result is a regenerate with a hypomorphic apical complex, with one article less than the normal one. A gradient of maturity persists during regeneration, but it is not tight, and it is more similar to that observable during comparably later developmental stages in normal development. We conclude that the positional information used by the epidermal cells that will produce a new article boundary and by those that will produce the setae is largely independent, and is not based on reciprocal signalling among these cells. Instead, the positional information that these cells use is determined along the proximo-distal axis of the flagellum/antenna and/or within a quartet.

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SEGMENTAL PATTERN FORMATION FOLLOWING AMPUTATION IN THE FLAGELLUM OF THE SECOND ANTENNAE OF *ASELLUS AQUATICUS* (CRUSTACEA, ISOPODA)[*]

ABSTRACT

Regeneration of the second antennae of *Asellus aquaticus* is described here following amputations along the antennal flagellum. The process involves the frequent resorption (loss of the distalmost joint remained on the amputated antenna) and the regular apicalization of the new terminal article. In the distal part of the flagellum, resorption occurs only when less than 70% of the original article length is left. For amputations performed in the proximal meristematic region, where new articles are normally produced, the new terminal article may also divide, sometimes producing articles with abnormal setal pattern; instead, articles that would normally divide may fail to do so if they are the nearest proximal neighbour of the new terminal article. Outcome of the increased production of new articles from the meristematic region is a regenerated antenna with a number of flagellomeres close to that shown by the undamaged controlateral one. Similarities and differences in respect to the processes occurring after amputation in the antennal peduncle, as well as in other arthropod limbs, are discussed. These differences may help understanding general properties of the regeneration process, such as the distinction between epimorphosis and morphallaxis and the relationship between normal development and regeneration.

INTRODUCTION

Regeneration has always attracted the interest of researchers, not only for its potential biomedical applications, but also for its evolutionary and developmental implications (e.g., GOSS, 1992; SÁNCHEZ ALVARADO, 2000, 2003, 2004; BROCKES *et al.*, 2001; GALIS *et al.*, 2003; BROCKES & KUMAR 2005; SÁNCHEZ ALVARADO & TSONIS, 2006). Many arthropods regenerate their appendages easily (reviewed in MARUZZO *et al.*, 2005), however these animals have not been adequately explored as experimental models in this regard. Due to the presence of serially homologous but regionally differentiated pairs of appendages, regeneration in arthropod offers also the possibility to study the phenomenon in different appendages (which may have different regeneration potential) within the same animal, as well as from different levels along the same appendage. Until now, however, most studies have been limited to the regeneration of the walking legs from the preferred breakage point (also called autotomy plane) (MARUZZO *et al.*, 2005).

The second antenna of the isopod *Asellus aquaticus* (Linnaeus, 1758) can provide a good model for studying the mechanism of production of new articles during regeneration in appendages different from legs. The uniramous second antennae of this crustacean are composed of a proximal six-article peduncle (WEGE, 1911) and a distal flagellum (Fig. 2.1). The flagellum is devoid of muscles (WEGE, 1911), and consists of articles (flagellomeres) increasing in number during the whole life of the animal. Using sensory setae as positional markers, MARUZZO *et al.* (2007) described in detail the postembryonic production of flagellomeres.

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[*] This paper is in press in the *Italian Journal of Zoology* (see p. 25): Diego Maruzzo, Mia Egredzija, Alessandro Minelli & Giuseppe Fusco. *Italian Journal of Zoology* (in press).

Most of the flagellum appears as a sequence of units of four articles (complete quartets), each article in a complete quartet being recognisable on the basis of a specific setal pattern. During postembryonic development, new quartets are produced from the proximal part of the flagellum, the meristematic region. The most basal flagellomere repeatedly gives off individual articles, each one being the founder of a future quartet (one-article incomplete quartet). A four-segmented, complete quartet will be formed after three divisions of the one-article incomplete quartet, passing through a two- and a three-article condition. Thus, the meristematic region is formed by the first flagellomere and a variable number of incomplete quartets of different types. It is followed by the central region of the flagellum which is composed of complete quartets, the number of which increases during postembryonic development. The distalmost articles (apical complex) are characterized by a peculiar setal arrangement. In antennae never incurred in regeneration, the apical complex always consists of five articles.

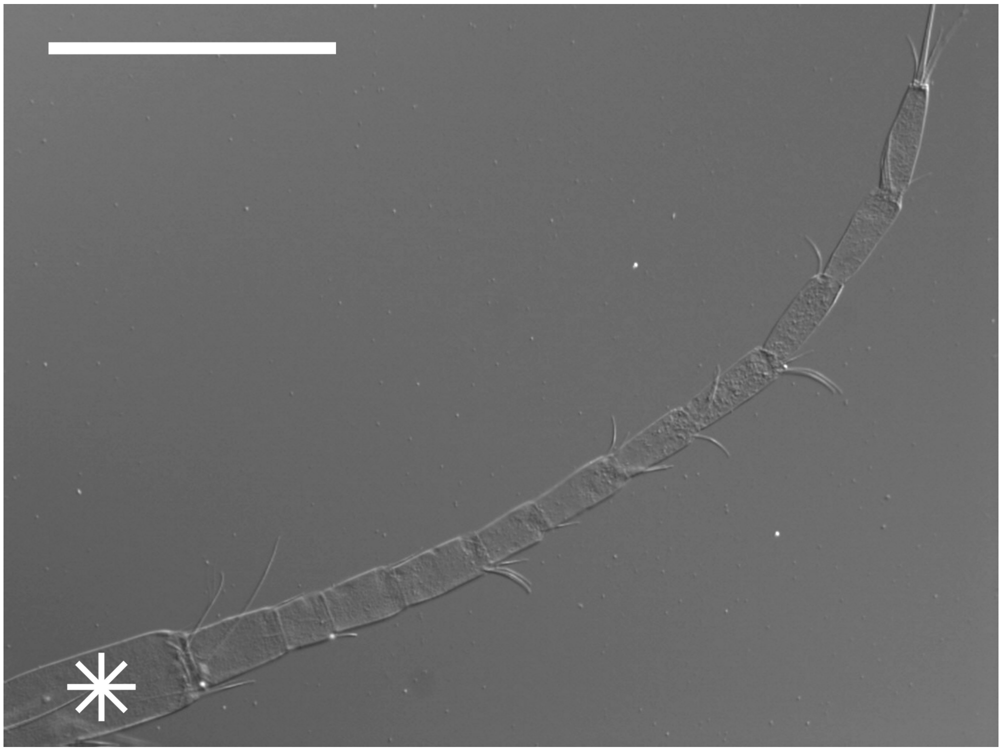

Fig. 2.1. The flagellum of a second antenna of a few days old *Asellus aquaticus* (exuvia observed with differential interference contrast). The most proximal article visible (*), in the lower left corner, is the distalmost article of the peduncle. Scale bar 200 µm.

MARUZZO *et al.* (2007) studied regeneration from the preferred breakage point which, in *A. aquaticus*, is placed between the fourth and the fifth article of the peduncle. During this kind of regeneration, the activity of the meristematic region increases, and there is always the formation of a specific “abnormal” apical complex of four articles. To investigate the relationship between the level of the amputation along the antenna and the pattern of regenerated articles, we present here data about regeneration following amputations performed at different levels along the flagellum (hereafter referred to as regeneration following flagellar amputations) using the same methodology (external morphology analysis) already used for studying the regeneration from the preferred breakage point.

MATERIALS AND METHODS

Isopods were collected and treated as described in MARUZZO *et al.* (2007). Briefly, specimens collected in a stream near Vicenza (Northern Italy) were reared in the laboratory. The specimens used in the experiments were kept in individual Petri dishes since the release from the female brood pouch. Amputations were performed on specimens anaesthetized for a short time with ice, using fine scissors or a microtome knife, under a dissecting microscope. The exuviae released by the amputated specimens were collected and studied. Observations on anaesthetized, living specimens were also performed.

Many variables could directly affect the detailed trajectory and the final outcome of the regeneration process. These are, for instance, the age of the specimen, the structure and number of flagellar articles, and the level of amputation. This latest variable can be further split in a number of subordinate variables, such as the level of amputation with respect the whole length of the flagellum (either in terms of metric distance or in terms of number of articles), the position of the amputated article within the quartet, and the level of the cut within the amputated article (the distance from the last join remained). We did not perform operations accounting for all possible combinations of these variables, as a complete inspection into the regenerative pathways of the flagellum was beyond the scope of the present investigation. We were instead more interested in the "generic" processes that occur independently of the effects of the above-mentioned variables, and indeed we could identify some of them. In our experiments we used young specimens (which moult and regenerate faster) and amputations were performed at different levels within the meristematic region (26 amputations in antennae with 12-20 flagellomeres) and the apical complex (40 amputations in antennae with 10-12 flagellomeres).

RESULTS

Two aspects will be considered separately in describing antennal regeneration following flagellar amputations: the production of a new apical complex and the further development of the flagellum. The production of a new apical complex is described here as the product of a few basic processes (see below), which are the same independently of the amputation level in the flagellum. The further development of the flagellum is the increase in the number of flagellomeres until the regenerating flagellum reaches approximately the same number of flagellomeres of the controlateral, undamaged antenna. This may happen in parallel to the production of a new apical complex, or require a few additional moults to be completed.

PRODUCTION OF A NEW APICAL COMPLEX

Apical complexes obtained by regeneration following flagellar amputations are far from uniform. Nevertheless, all these apical complexes are the result of different combinations of few basic processes: apicalization, resorption, incomplete division of a quartet, and division of the (new) terminal article.

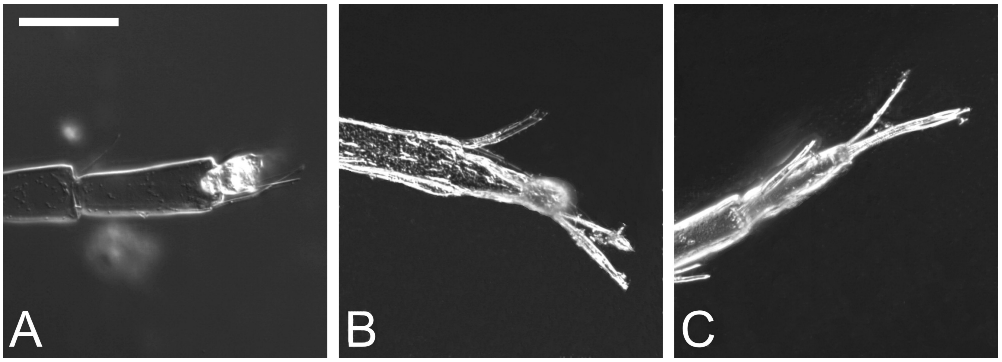

Fig. 2.2. Apicalization of an article. A, first exuvia released after the amputation. B, second exuvia released after the amputation of the same antenna showing the most distal complete article in A going through apicalization (changes in size and shape). C, third exuvia released after the amputation, showing almost complete apicalization (setal pattern will slowly change further with the following moults). Pictures are overlays of different focus-level photographs obtained with differential interference contrast. Scale bar 50 µm.

Apicalization. This process occurs always, irrespectively of the amputation level in the flagellum. Apicalization is the transformation into a new apex of the distalmost article left after the amputation (Fig. 2.2). This transformation involves both the shape of the article and the number and arrangement of its setae. It was already known from old histological studies that the first article to be formed anew during regeneration is always the distalmost one (WEGE, 1911).

Resorption. This process may occur following amputations performed at any level along the flagellum. When it occurs, the joint between the last article left after the amputation (usually a stump) and its most proximal neighbour is lost and the stump is incorporated into the proximal article (Fig. 2.3). For amputations in the meristematic region, this mainly occurs when a short article stump is left, but, although less frequently, even resorption of an almost complete article has been recorded. For amputations in the apical complex the occurrence of resorption phenomena depends on more specific conditions. When a stump longer than 70% the whole article length is left (this was measured in the first exuvia obtained after the amputation, using the length of the corresponding article of the controlateral, undamaged antenna as term of comparison), the article stump is not incorporated into the adjacent article. Instead, resorption occurs when less than 70% of an article length is left. After resorption, the newly formed terminal article always undergoes apicalization (Fig. 2.3).

Incomplete division of a quartet. This is the case when the incomplete quartet developing close to the newly formed apex does not develop completely. For example, if a two-article incomplete quartet is just proximal to the newly apicalized article, the distal member of this incomplete quartet may remain undivided, thus forming an incomplete, three-segmented "quartet"; morphologically, it then resembles the condition where the equivalent of the third article of a complete quartet is missing (see Fig. 2.4). This outcome was observed rarely (5 cases), and was obviously limited to amputations in the meristematic region.

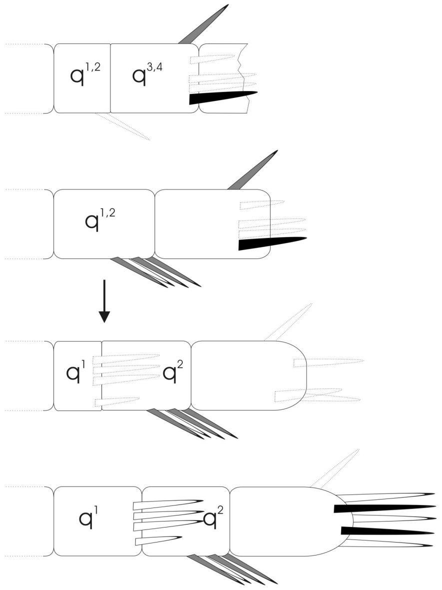

Fig 2.3. Schematic representation of an example of resorption. A left antenna is illustrated; distal (apical) to the right; proximal articles omitted; dorsal setae are represented in black, lateral setae in grey, ventral setae in white; dashed setae may be present or not. Number and location of setae on the distalmost article (apex) show great variability, the pattern shown in the figure is an example. Here, the amputation has left an article stump distal to a two-article incomplete quartet (articles $q^{1,2}$ and $q^{3,4}$); the joint between the stump and the adjacent proximal article gets incorporated and the resulting distalmost article goes through apicalization. At the same time the proximal article ($q^{1,2}$) of the original two-article incomplete quartet divides, producing the two proximal articles (q^1 and q^2) of a complete quartet; arrow points at the plane of division.

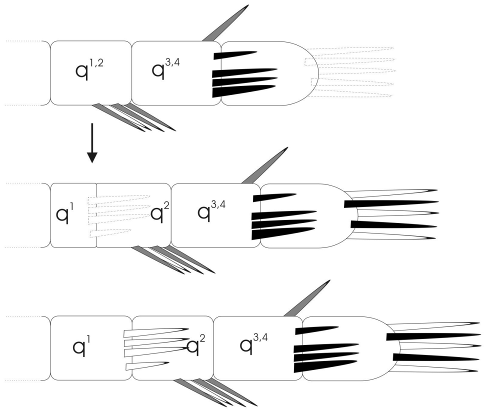

Fig. 2.4. Schematic representation of an example of incomplete division within a quartet. Orientation and labels as in Figure 1. In this example a two-article incomplete quartet (articles $q^{1,2}$ and $q^{3,4}$) just proximal to the newly apicalized article develops into an abnormal three-segmented quartet, as the distalmost article ($q^{3,4}$) does not divide. This missing division leaves an article morphologically undistinguishable from a q^4, indicated here as $q^{3,4}$ to highlight its segmental origin. Arrow points at the plane of division.

Division of the (new) terminal article. Following amputations in the meristematic region, the new terminal article may also divide; indeed, this process has been recorded only (but not always) when just two or three flagellomeres were left. The result of this division is different according to the degree of apicalization reached by this article at the time of division. Division of an article which is morphologically recognizable as apex (even though it does not bear yet a complete set of setae), gives rise to an article with the characteristic morphology of the subapical article in a normal apical complex (see Fig. 5 in Maruzzo *et al.*, 2007[*]). In contrast, division of an article that was going to become the apex, but had not yet assumed the typical morphology, produces articles with abnormal setal pattern.

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[*] Fig. 1.5 p. 37.

A summary of the different apical complexes recorded in our experiments is given in Table 2.1.

FURTHER DEVELOPMENT OF THE FLAGELLUM

Comparing antennae amputated in the meristematic region with antennae amputated in the apical complex, there are differences in the further development of the flagellum. This is expected on the basis of a simple quantitative considerations: in fact amputations at the level of the apical complex remove just a few articles, whereas those in the meristematic region remove most of the flagellum. In the latter case, the developmental activity related to the meristematic region differ from the normal morphogenesis in the same way as described for amputations at the level of the preferred breakage point, i.e., all new articles are produced in the meristematic region, which markedly increases its rate of article production (MARUZZO *et al.*, 2007). After amputations in the apical complex, no significant differences in the processes related to the meristematic region have been usually observed, in comparison with what happens in undamaged antennae. Indeed, in most cases, after the production of a new apical complex, the amputated antenna remains with one or two articles less than the controlateral, undamaged one. Comparable levels of left-right asymmetry in the undamaged antennae of the same specimen are also often found (unpublished data).

However, in 2 cases out of 40, we found evidence for the production of new articles even in the distalmost part of the flagellum, through unusual division of articles close to the apex. These are the only data showing production of new articles outside the meristematic region, since also the division of the apex (see above) has been observed only when the antenna was amputated in the meristematic region, i.e., when the dividing apicalized article was itself an article of the meristematic region. These two individual cases will not be discussed further here, in the absence of additional evidence, although an abnormal case briefly described by WEGE (1911) could also belong here.

DISCUSSION

OCCURRENCE OF APICALIZATION AND RESORPTION OF ARTICLES

While apicalization occurs always, both in complete articles and article stumps, resorption does not. Amputations in the apical complex are followed by resorption only when less than 70% of the damaged article is left (as measured on the exuvia, the actual damage of the internal tissues being unknown). Amputations in the meristematic region does not show a consistent pattern. The articles of the meristematic region are very short, so a small difference in length can be large in relative terms. Also, small differences in the amount of damage in the internal tissues, which cannot be evaluated from our data, could be significant.

A similar resorption of joints was described in the regeneration of the cockroach leg. In this case, regeneration from the preferred breakage point (which is here between the second [trochanter] and the third [femur] leg article) involves cell movement and division not only in the trochanter, but also in the coxa, i.e. in the article proximal to it. The coxa-trochanter joint is thus lost and reformed during regeneration (Truby, 1983, 1985), whereas in the regenerating antennae of *Asellus* the lost joints are not produced again.

Table 2.1. Summary of the apical complexes emerging from normal development (undamaged antennae), regeneration from the preferred breakage point (PBP), and regeneration following flagellar amputation. q^1, q^2, q^3, and q^4 indicate articles with the setal pattern typical of, respectively, the first, second, third, and fourth article (in proximo-distal order) of a complete quartet (see Maruzzo *et al.*, 2007). Articles q^1 and q^3 have a very similar setal pattern (see figures in Maruzzo *et al.*, 2007; also, discussion in Racovitza, 1925), and their distinction is often based on relative position only; $q^{1/3}$ indicates an article that according to the setal pattern could be identified as either q^1 or q^3. In subapical position, a $q^{1/3}$ can be either produced by division of the new terminal article or be already present (as q^1 or q^4) but becoming subapical only after apicalization of the next article distal to it. Formulas in square brackets specify the origin of these articles: q^{subA} is an article with setal pattern $q^{1/3}$, but produced by the division of the new terminal article; x^{subA} is an article with abnormal setal pattern produced by the division of the new terminal article; $q^{3,4}$ is the distal member of a two-article incomplete quartet; $q^{1,2,3,4}$ is a one-article incomplete quartet, i.e., a potential quartet founder, as released from the meristematic region (both $q^{3,4}$ and $q^{1,2,3,4}$ exhibit the setal pattern of q^4; see Fig. 2.2). Data for the normal apical complex and the apical complex produced after regeneration from the preferred breakage point (for both of which no variability was recorded) are from Maruzzo *et al.* (2007); the ontogeny of the normal apical complex is based on the hypothesis presented in this paper (see text and Fig. 2.3). The number of observed cases is shown. The two specimens which showed production of new articles also in the distalmost part (see text) are not included.

Developmental context	Composition of the apical complex		Number of observed cases
Normal development	$q^1\ q^2\ q^4\ q^{1/3}$ Apex	[$q^1\ q^2\ q^{3,4}\ q^{\mathrm{subA}}$ Apex]	see Maruzzo *et al.* (2007)
Regeneration from PBP	$q^1\ q^2\ q^{1/3}$ Apex	[$q^1\ q^2\ q^{\mathrm{subA}}$ Apex]	see Maruzzo *et al.* (2007)
Regeneration following flagellar amputation (N=64)	$q^{1/3}$ Apex	[q^1 Apex]	12
		[q^{subA} Apex]	4
	$q^1\ q^2$ Apex		15
	$q^1\ q^2\ q^{1/3}$ Apex	[$q^1\ q^2\ q^{\mathrm{subA}}$ Apex]	8
		[$q^1\ q^2\ q^3$ Apex]	3
	Apex		9
	$q^1\ q^2\ q^4$ Apex	[$q^1\ q^2\ q^{3,4}$ Apex]	7
	x Apex	[x^{subA} Apex]	3
	$q^1\ q^2$ x Apex	[$q^1\ q^2\ x^{\mathrm{subA}}$ Apex]	1
	$q^1\ q^2\ q^4\ q^{1/3}$ Apex	[$q^1\ q^2\ q^{3,4}\ q^{\mathrm{subA}}$ Apex]	1
	$q^1\ q^2\ q^4\ q^4$ Apex	[$q^1\ q^2\ q^4\ q^{1,2,3,4}$ Apex]	1

INCOMPLETE DIVISION OF A QUARTET AND DIVISION OF THE TERMINAL ARTICLE

We observed articles with an abnormal setal pattern only in antennae regenerating from the flagellum and always as the result of the division of an article that was going to become the apex, although not yet morphologically differentiated. RACOVITZA (1925) described specimens from field collections showing flagellomeres with abnormal setal pattern, and these were always adjacent to the apex, except for one case. In this case, the specimen presented also other unusual features that can be interpreted, as was suggested by RACOVITZA (1925), as the result of repeated regeneration processes following flagellar amputations. In contrast, the division of an already apicalized article produces proximally an article with the typical setal pattern of the normal subapical article.

The results shown here may suggest a mechanism through which the normal apical complex can be developed, by incomplete division of a quartet followed by the additional division of the terminal article. This hypothesis is schematically presented in Fig. 2.5.

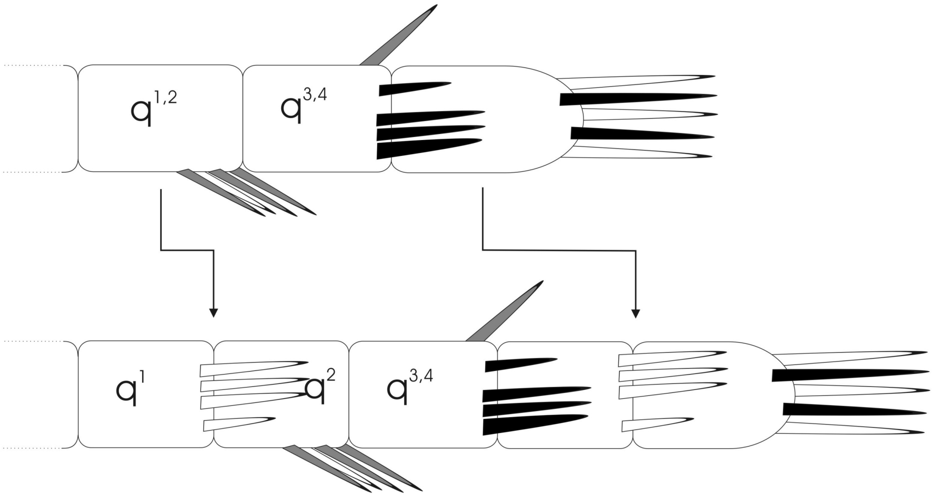

Fig. 2.5. Hypothetical reconstruction of the normal development of the apical complex. Orientation and labels as in Figure 1. Starting with a two-article incomplete quartet (articles $q^{1,2}$ and $q^{3,4}$) just proximal to the apex, the hypothesis involves incomplete division of the incomplete quartet (as in the example in Fig. 2.4), plus division of the apex. Arrows point at the planes of division.

ACTIVITY OF THE MERISTEMATIC REGION FOLLOWING FLAGELLAR AMPUTATION

As noted above, the activity of the meristematic region following amputation in the proximal part of the flagellum is different from its activity following more distal amputation. While in the latter case the segmentation process is not affected, in the former case it changes in a manner comparable to the segmentation process that follows an amputation in the peduncle. Our observations are comparable to those reported by SCHAFER (1973) for antennal regeneration in the cockroach. During regeneration following amputation in the proximal part of the flagellum, the proximal meristematic region increases the rate of article production in respect to normal development, whereas amputations distal to the tenth flagellomere does not seem to change the morphogenetic activity of the meristematic zone (SCHAFER, 1973).

IS THIS REGENERATION?

It is generally acknowledged that crustaceans regenerate limbs by producing an apical epithelial cup and a mass of dedifferentiated cells (the blastema) which grow and reproduce the lost limb (e.g., NEEDHAM, 1965; ADIYODI, 1972; HOPKINS *et al.*, 1999). Most studies, however, are based on autotomized legs. Regeneration from different levels along the flagellum, as described here, is different. After apicalization (and eventually resorption, incomplete division of a quartet and division of the terminal article), the damaged flagellum approaches the full length and segmentation of the controlateral one just by increasing growth and segmentation rates. Apicalization seems to involve some cell division and differentiation (WEGE, 1911), and resorption is likely to involve some dedifferentiation as well (cells that previously produced an article boundary do not maintain it any longer). Although some form of control specific of the regeneration process may exist, both the incomplete division of a quartet and the division of the terminal article are possibly the outcome of the normal developmental process acting under different positional information condition. The increased rates of growth and segmentation are then stopped by an unknown form of control on both size and article number (which are strictly correlated; unpublished data) in the regenerating antenna.

Most of the regeneration of the flagellum of the second antennae of *A. aquaticus*, which has indeterminate growth and article addition, is thus achieved by increasing the rate of the normal morphogenetic process (see above). In general, the relationships between regeneration and the widespread replacement of cells during normal physiological turnover are currently far from clear (see, e.g., SÁNCHEZ ALVARADO, 2003). In flatworms, regeneration is achieved by the proliferation of stem cells (neoblasts) which are the animal's only mitotically active cells and in undamaged animals provide replacement for the cells that go lost during the worm's lifetime (e.g., NEWMARK & SÁNCHEZ ALVARADO, 2000). At present we do not have data for comparing the dynamics of the epidermis during normal development with those during regeneration in the model we studied, but in both cases mitosis are probably localized in the basalmost part of the flagellum. If so, the mechanism for which new cells are added during postembryonic development would be the same during regeneration.

Amputations in crab legs or even in the peduncle of the second antennae of *A. aquaticus* lead to a slightly different regeneration. In both the crab leg and the isopod's antennal peduncle there is no increase in article number during postembryonic development and no specific growth region seems to exist. In these cases, regeneration involves extensive cell migration, dedifferentiation and division at the level of amputation and the new peduncle or leg grows out there (WEGE, 1911; ADIYODI, 1972; HOPKINS *et al.*, 1999).

Indeed, it has already been noted (NEEDHAM, 1965) that the distal region of multisegmented appendages (such as isopod or insect antennae) usually regenerates differently from other limbs or even from part of the same limb with mesodermal components (muscles), and this was related to a bigger degree of morphallaxis (cellular remodelling) than epimorphosis (cellular division and dedifferentiation) for the former parts. Thus, while the distinction between epimorphosis and morphallaxis is widely used in discussing regeneration (e.g., SÁNCHEZ ALVARADO, 2000, 2003; BROCKES *et al.*, 2001; GILBERT, 2003), this is not a sharp division (see also AGATA *et al.*, 2007), and the second antennae of *A. aquaticus* may provide a nice model for studying the interplay and limits of these definitions. A lack of a sharp distinction between the two phenomena should not be surprising, however, as it was already clear to MORGAN (1901), when he first introduced the distinction between epimorphosis and morphallaxis.

For a better understanding of the generative processes of form, we want to stress the importance of not taking for granted some traditional and somehow arbitrary distinction (such as epimorphosis/morphallaxis and normal development/regeneration). A more specific focus on the cell-level processes and their many combinations seems indeed more promising.

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COMPARATIVE MORPHOLOGY AND DEVELOPMENT OF ISOPOD (CRUSTACEA) SECOND ANTENNAE

ABSTRACT

Setal distribution pattern and mechanism of production of new articles in the second antennal flagellum of four isopod species (*Asellus aquaticus*, *Lirceus fontinalis*, *Sphaeroma serratum* and *Idotea chelipes*) is described and compared. For all these species the first flagellar article (called meristematic) is the main producer; articles produced by it may also go through further divisions, the number of which depends on the specie: three in *Asellus*, one in *Lirceus* and *Sphaeroma* and none in *Idotea*. The utility of studying comparative morphology and development of the antennal flagellum for both phylogenetic and evolutionary studies is discussed.

INTRODUCTION

The second antennae of isopod crustaceans are usually described as composed of a proximal peduncle and a distal flagellum. While the peduncle is composed of articles with intrinsic muscles, the flagellar articles (usually called annuli) and completely devoid of muscles, i.e., the last muscle(s) of the second antenna have their terminal insertion at the joint between the peduncle and the flagellum. The flagellum of isopod second antennae has never been studied in great detail, especially in a comparative way. This is mainly because isopod comparative morphologists have been mainly taxonomists, and the second antennal flagellum provides very few useful characters for taxonomic purpose. Some papers deal with the peduncle of the isopod second antennae: SCHMALFUSS (1974) and SCHELOSKE (1977) provided detailed comparative anatomical observation, WÄGELE (1983) discussed problems in the distinction between peduncle and flagellum through external morphological analysis, and BRUSCA & WILSON (1991) provided an overview of the external morphology (mainly, the number of articles) of the peduncle across all major isopod groups. Regarding the flagellum there are only few papers that deal with the morphology and evolution within some isopod groups. For oniscid isopods, HOESE (1989) and SCHMALFUSS (1998) provided interesting observations; however, they focussed mainly on functional morphology, and did not consider other aspects such as setal distribution pattern and mechanism of article production (in the following, segmentation) in the flagellum.

The most accurate work performed on the comparative morphology of the second antennal flagellum, taking setal pattern distribution and segmentation into account, is RACOVITZA (1925) on asellid isopods. His work was later completed, with more precise observations on normal development and regeneration, by MARUZZO *et al.* (2007, in press) on the asellid *Asellus aquaticus* (Linnaeus, 1758). RACOVITZA (1925) studied several species and, among them, he described only one genus, *Lirceus* (sub *Mancasellus*), which does not exactly align to what is described for other asellids. Information on the segmentation of the antennal flagellum is also available for valviferan isopods (see NAYLOR, 1955 for several species of *Idotea* and EL HEDFI-BEL HAJ KHELIL, 2002 for *Synisoma nadejda* Rezig, 1989). However, no specific description of the setal distribution pattern is available for species of this group. For other isopod groups, neither the segmentation nor detailed descriptions of setal distribution pattern are available for comparative purpose.

Here I provide some observations, with both light and scanning electron microscopy (SEM), on the second antennal flagellum of two species for which some information on both setal distribution pattern and segmentation are already available (*Asellus aquaticus* and *Lirceus fontinalis* Rafinesque-Schmaltz, 1820; both Asellidae), one specie for which information on segmentation but not on setal distribution pattern is available (*Idotea chelipes* (Pallas, 1766); Idoteidae), and one specie for which information on neither setal distribution pattern nor segmentation is available (*Sphaeroma serratum* (Fabricius, 1787); Sphaeromatidae). These data are then discussed in a comparative way and the usefulness of this kind of observations for both phylogenetic and evolutionary studies is outlined.

MATERIALS AND METHODS

Stored specimens of *A. aquaticus* from previous studies (MARUZZO *et al*., 2007, in press) as well as some new material from the same site (Vicenza, Italy) were used. Specimens of *L. fontinalis*, collected near Bloomington (Indiana, USA), were kindly provided by Dr. J.J. Lewis and S.L. Lewis. Specimens of *I. chelipes* and *S. serratum*, both collected in the Venetian lagoon (Italy), were kindly provided by Dr. M. Rampin. Specimens were fixed and stored in either 4% paraformaldehyde in PBS, 70% or 90% ethanol. For light microscopy the specimens (except the smaller ones) were digested in KOH and incubated for 10 minutes in glacial acetic acid. All the specimens were mounted in glycerol and observed in bright field or differential interference contrast (DIC). For SEM observations specimens were dehydrated in a graded ethanol series, dried with hexamethyldisilazane (Sigma) (NATION, 1983), and coated with gold. Examinations were made with a Cambridge Stereoscan 260 or with a Jeol JSM-6490 scanning electron microscope.

RESULTS

ASELLUS AQUATICUS

For this specie both setal distribution pattern and segmentation have been already described (RACOVITZA, 1925; MARUZZO *et al.*, 2007); the purpose of this section is to briefly review this knowledge and to provide SEM picture that can be directly compared with those of the other species.

In *A. aquaticus* most of the flagellum is composed of quartets, series of four articles each one of which bears a distinctive setal pattern (Fig. 3.1; the whole section of the flagellum composed of quartets is called central region). The flagellum keeps on acquiring new articles (and quartets) during the whole postembryonic development. All new articles and quartets are produced in the proximal part of the flagellum (called meristematic region): the first article (the meristematic article) keeps on giving off new articles; each of these (called one-article incomplete quartet) in turn divides - rather independently from others - three more times, producing four final articles (a complete quartet) (Fig. 3.2, 3.3). The distalmost part (called apical complex) is not arranged in quartets; while an undamaged flagellum has always a five-articles apical complex (see Fig. 5 in

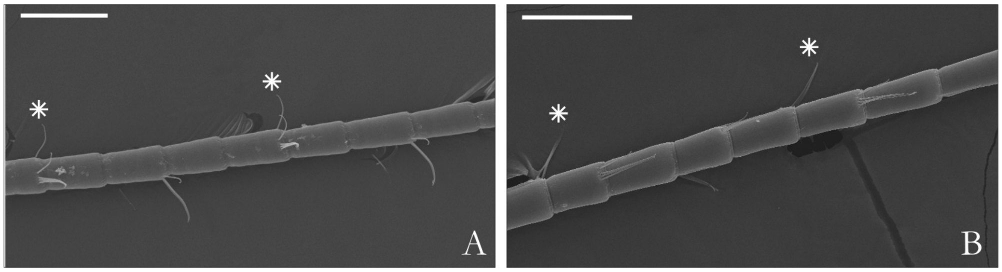

Fig. 3.1. *Asellus aquaticus*, some articles from the central region of two flagella. SEM. Proximal to the left; the setae that lies on the outer side, which mark the end of a complete quartet (see Fig. 3.2), are marked by *. Note that the same setal pattern repeats every four articles. A, dorsal view of a left flagellum. B, ventral view of a right flagellum. Scale bars, 100 µm.

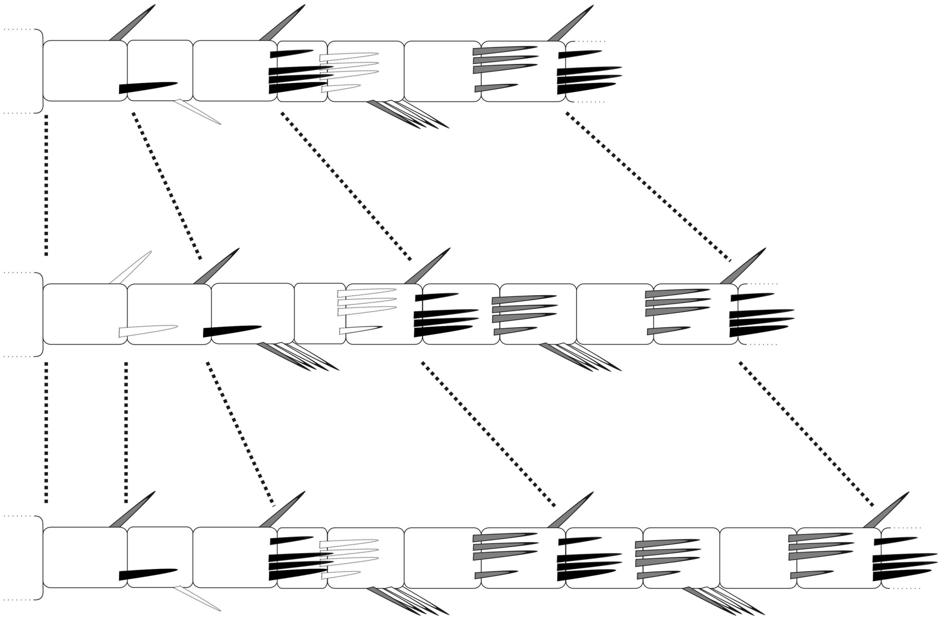

Fig. 3.2. Three stages in the production of new articles in *Asellus aquaticus*. Only the proximal part of a flagellum is considered; proximal to the left, distal to the right; outer side above, inner side below; black setae are dorsal, white ventral, grey lateral; dashed setae may or may not be already present. The grey setae on the outer side correspond to those marked by * in Fig. 3.1.

MARUZZO *et al.*, 2007[*]), following regeneration from the peduncle a four-article apical complex is always obtained (MARUZZO *et al.*, 2007) and following amputations along the flagellum many different kind of apical complexes can be produced (MARUZZO *et al.*, in press).

LIRCEUS FONTINALIS

RACOVITZA (1925) described setal distribution pattern and segmentation from a few specimen of *L. fontinalis* (sub *Mancasellus macrourus*); our observation

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[*] Fig. 1.5 p. 37.

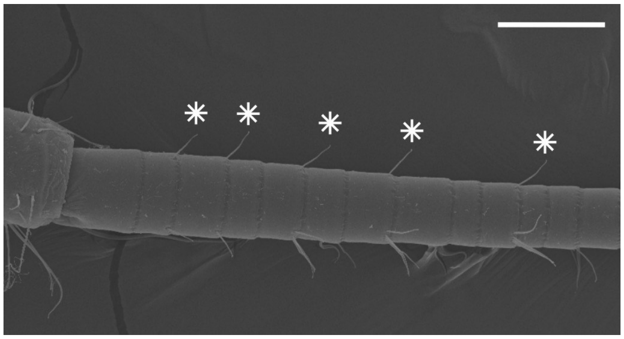

Fig. 3.3. *Asellus aquaticus*, the meristematic region of a flagellum. SEM. Orientation as in Fig. 3.1A. Setae marked by * correspond to those marked in the same way in Fig. 3.1. Except for the most proximal article of the flagellum (the meristematic article), all of the others are part of incomplete quartets (quartets that by further divisions will become complete, i.e., four-segmented). Scale bar, 100 µm.

mostly confirm his interpretation. In *L. fontinalis* the four different setal patterns characteristic of four articles in *A. aquaticus* are still recognizable, but are distributed on two articles only; thus most of the flagellum has repetitively in setal pattern every two articles, not every four (Fig. 3.4).

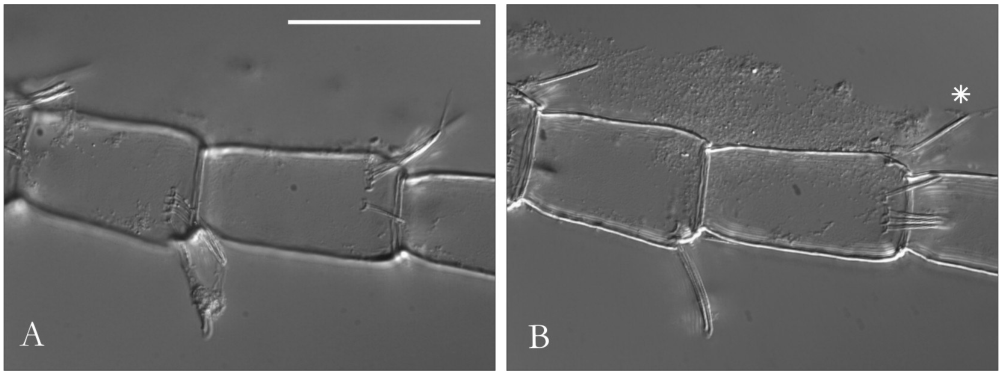

Fig. 3.4. *Lirceus fontinalis*, central part of a flagellum. Overlay of different focus-level pictures obtained with DIC of a specimen digested with KOH; proximal to the left, outer side above. A, overlay of pictures showing the ventral side, the two rows of setae correspond to those of the first and third article of a quartet in *A. aquaticus* (i.e., the white setae in the distalmost quartet of Fig. 3.2). B, the same articles but in an overlay of pictures showing the dorsal side, the two rows of setae correspond to those of the second and fourth article of a quartet in *A. aquaticus*; * marked seta homologous to those marked in Fig. 3.1. Scale bar, 100 µm.

The segmentation is also comparably similar to that of *A. aquaticus*: the first flagellar article produces new articles that will divide only one more time (rather than three times as in *A. aquaticus*) producing two final articles.

The flagellum of *Lirceus* shows, however, some variation both in the number of setae and in the segmentation process. In *Asellus* the two ventral rows of setae on the first and third articles of a quartet, have always composed of just four setae. In *Lirceus*, while this is also the most common number for the corresponding rows, higher number are not uncommon and sometimes two separate rows, at different level along the proximo-distal axis of an article, are even produced (Fig. 3.5A). Concerning segmentation, while a two-articles repetitively is usually found, triplets and quartets (just as in *Asellus*) also sometimes found (Fig. 3.5B).

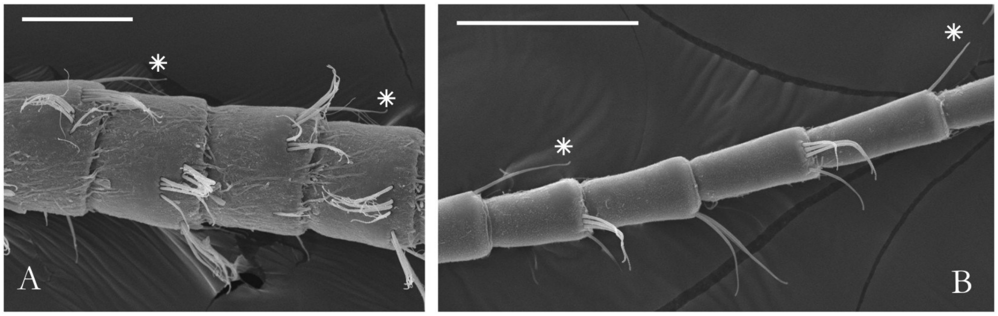

Fig. 3.5. *Lirceus fontinalis*, central part of two flagella. SEM. Ventral view, proximal to the left, outer side above. The setae marked with * are homologous to those marked with the same symbol in *A. aquaticus* (see Fig. 3.1). A, a two-article pattern; note the high number of setae on the ventral side that appear as two separate rows. B, a "quartet" very similar to those in *Asellus* (compare it with Fig. 3.1B). Scale bars, 100 µm.

SPHAEROMA SERRATUM

Most of the flagellar articles in *S. serratum* have pretty uniform setal pattern: some tufts of four setae on the inner side (Fig. 3.6A) and one single seta, of different morphology, on the outer side (Fig. 3.6B). The actual number of tufts, which is usually four, appears to increase in older article; the maximum number observed was five.

It was already known that during postembryonic development the number of articles in the antennal flagellum of this species increases (PIGEAULT-DAGUERRE DE HUREAUX, 1959). New articles are produced in the proximal part of the flagellum by division of the first and second article of the flagellum (Fig. 3.7).

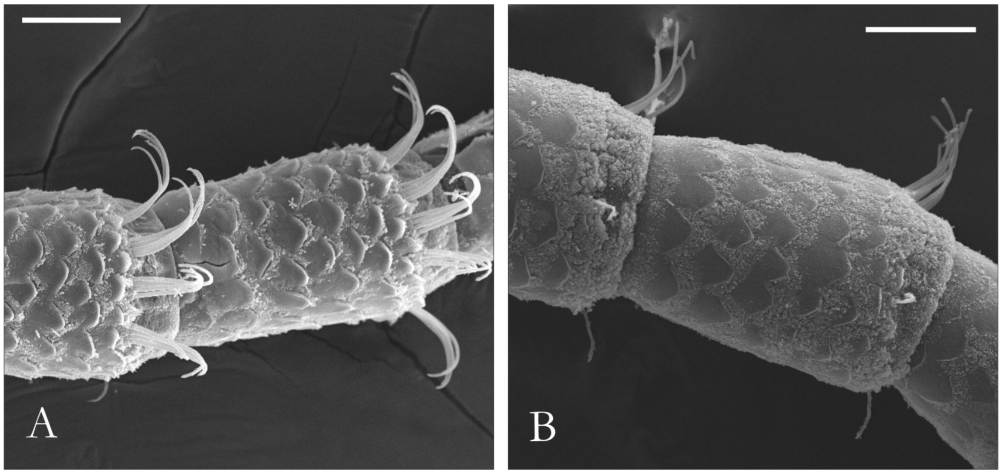

Fig. 3.6. *Sphaeroma serratum*, the central part of two flagella. SEM. A, inner side of a flagellum, proximal to the left, upper side is ventral. B, outer side of a flagellum, proximal to the left, upper side is dorsal. Scale bars, 50 µm.

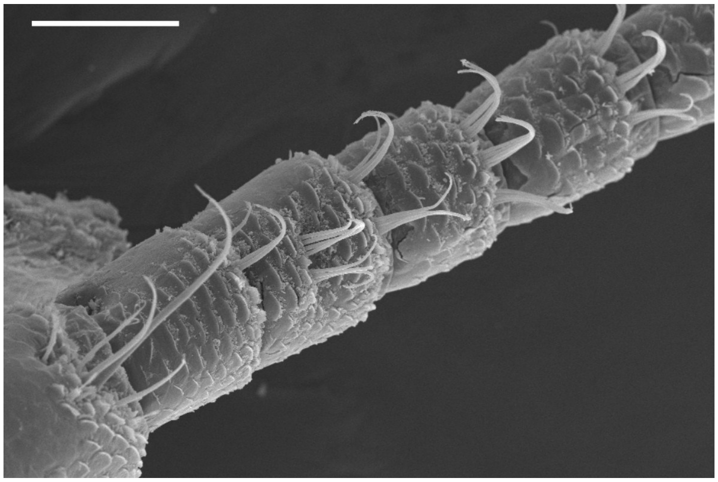

Fig. 3.7. *Sphaeroma serratum*, proximal part of a flagellum. SEM. Inner side of a left flagellum, proximal to the left, upper side is dorsal. The second flagellar article has not completed a division. Scale bar, 100 µm.

IDOTEA CHELIPES

In *I. chelipes*, most of the flagellar articles have subequal setal pattern: some tufts of four setae all around the joints (Fig. 3.8). The actual number of tufts, which is usually five, appears to increase in older article; the maximum number observed was six.

As it was already known (NAYLOR, 1955), new articles on the antennal flagellum are only produced by the division of the most proximal one.

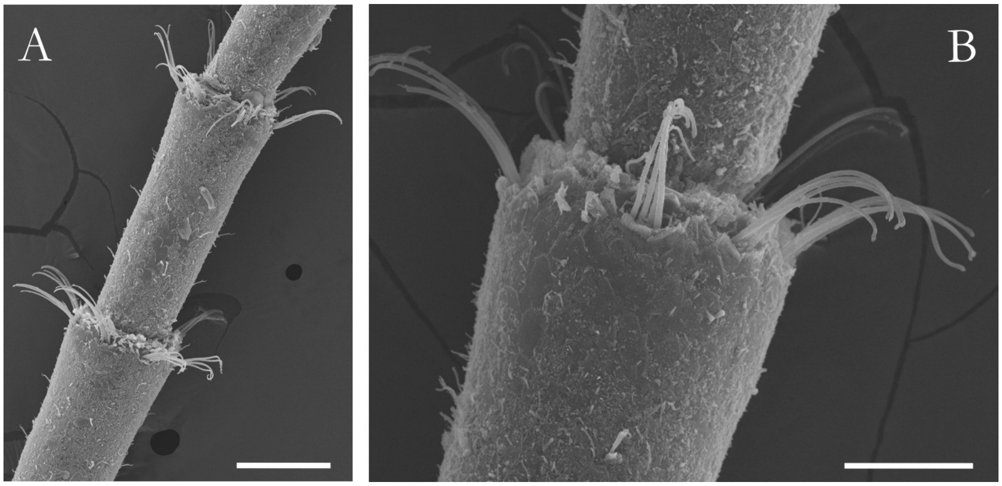

Fig. 3.8. *I. chelipes*, central part of the flagellum. SEM. Ventral view of a left flagellum, proximal part below. A, general view; scale bar, 100 µm. B, higher magnification on some tufts of setae; scale bar, 50 µm.

DISCUSSION

ANTENNAL DEVELOPMENT IN ISOPODS

MARUZZO *et al.* (2007) discussed the comparative development of isopod antennae taking into account their data on *Asellus aquaticus* as well as data available from literature. In this paper I re-examinated three species, *A. aquaticus*, *L. fontinalis* and *I. chelipes*, for which knowledge on the segmentation were already available; our data basically confirm previous reports, with just some more observation to be added only for *L. fontinalis*. In *Lirceus* the articles produced by the meristematic (more proximal) one usually divide just once; sometimes, however, they divide three and even four times, reproducing the condition found in *Asellus*. Both RACOVITZA (1925) and MARUZZO *et al.* (2007) supported the idea that the *Lirceus* condition is derived from an *Asellus*-like one, and their arguments will not be repeated here; the variation just described shows that the developmental control of segmentation is not so "strong" and the antennal flagellum of *L. fontinalis* appears as an interesting model for more specific studies on the evolution of the segmentation mechanism in the antennal flagellum.

I here also studied a species, *S. serratum*, for which the segmentation of the antennal flagellum was not described. The mechanism is basically identical to the *Lirceus*' one but, while in later the flagellum ends up with a two-article repetitivity, in *Sphaeroma* it ends up with just one-article repetitivity.

All these data confirm what was already noted (MARUZZO *et al.*, 2007): the first flagellar article is always the main producer but there is specific variation in the number of division the articles produced by the first one get through. The variation in the number of divisions of the articles produced by the meristematic one, however, is not necessarily mirrored by the degree of article-number repetitivity in the central part of the flagellum, although most of the times is.

More comprehensive studies on isopod antennal flagellum development appear promising not only to understand the evolution of flagellar segmentation, but also for studying the evolution of the mechanism controlling production and position of setae.

COMPARATIVE MORPHOLOGY OF ISOPOD SECOND ANTENNAE

The second antennal flagellum of isopods has never been subject of a comparative analysis. Only RACOVITZA (1925) made an attempt for asellid isopods; he dared to do that, however, only after understanding the basic mechanism of article production and comparing what he considered to be the "fundamental unit", namely, the quartet ("section" in his terminology). Making comparisons taking into account the repetitivity of the setal pattern distribution seems indeed promising for understanding the evolution of isopod second antennal flagellum (Fig. 3.9). However, for doing that across all isopods an isopod phylogeny is needed. Until now there are just a few published works attempting to resolve the phylogenetic relationships of all major isopod groups (WÄGELE, 1989; BRUSCA & WILSON, 1991; TABACARU & DANIELOPOL, 1998), but none of these works found acceptance consensus and new problems are emerging meanwhile (see, e.g., WETZER, 2002; BRANDT & POORE, 2003; WÄGELE *et al.*, 2003).

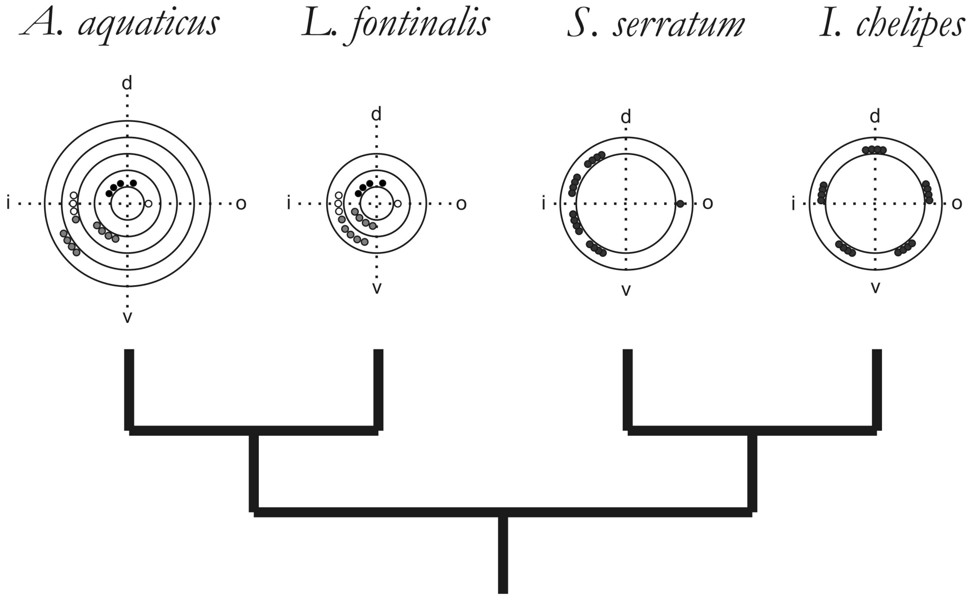

Fig. 3.9. Phylogenetic relationships and the setal pattern of the species studied. The setal pattern is shown as vertical projection: each circumference correspond to a joint, the smaller the circumference, the distal the article; dots represent setae. d, dorsal side; v, ventral side; i, inner side; o, outer side; setae are in black, white or grey in *Asellus* and *Lirceus* to maintain correspondence with Fig. 3.2, setae of *Sphaeroma* and *Idotea* are all in dark grey. Phylogenetic relationships are from BRUSCA & WILSON (1991) and DREYER & WÄGELE (2002).

Data on comparative morphogenesis and morphology can be used, anyway, as characters for constructing a phylogeny or even for stimulating new phylogenetic hypotheses. I provide here just one example regarding phreatoicidean isopods. Phreatoicidea have been placed close to many different groups and in many different positions within Isopoda by different authors. The works of WÄGELE (1989), BRUSCA & WILSON (1991) and TABACARU & DANIELOPOL (1998) all agree on a basal position of phreatoicideans, as sister-group to all other isopods, followed by the Asellota, i.e., [Phreatoicidea [Asellota [other isopods]]]. However, subsequent analysis showed that different dataset and methods yielded different results (e.g., WILSON, 1998; DREYER & WÄGELE, 2001, 2002; WETZER, 2002) and phreatoicideans have been also recovered as: 1) sister-group to all other isopods except for Asellota, which were sister-group to all other isopods including phreatoicideans, i.e., [Asellota [Phreatoicidea [other isopods]]]; 2) sister-group of the Asellota, with this monophyletic group sister group of all other isopods, i.e., [[Phreatoicidea, Asellota] other isopods]. By studying published pictures of the antennal flagellum of different phreatoicideans it seems that the monophyletic status of this group can be questioned. *Eremisopus beei* Wilson & Keable, 2002 has flagellar articles with a setal pattern very similar to that shown here for *Idotea*: articles with tufts of setae all around a joint, and each article very similar to the others (see Fig. 2D of WILSON & KEABLE, 2002). *Platypyga subpetrae* Wilson & Keable, 2002 seems to have a slightly different condition, with tufts not so clear as in *E. beei* and a possible repetitivity every two articles (see Fig. 16F of WILSON & KEABLE, 2002). No clear tufts, but all articles with the same setal pattern, seems also to be present in *Crenisopus acinifer* Wilson & Keable, 1999 (see Fig. 3D in WILSON & KEABLE, 1999). *Colubotelson* *thomsoni* Nicholls, 1944 seems to have a very different condition. For this specie it is not known on how many article a similar setal pattern is found since there is just a SEM picture of a single article available, but this article shows a setal pattern apparently identical to the one of the distalmost article of *Asellus*' quartet (see Fig. 11 of HOESE, 1989). As far as I know the monophyletic status of Phreatoicidea has never been questioned, but it has also never been tested cladistically. The different morphologies of the antennal flagellum I have just summarized, however, strongly invite testing the group's monophyly.

In this paper I dealt only with the proximal and central part of the flagellum. In *Asellus* the distalmost part (called apical complex) is not arranged in quartets and, following regeneration, its structure can deeply change, an aspect that does not apply to the proximal and central region (MARUZZO *et al.*, 2007, in press). In principle, also the distalmost part can be interesting as much as the central part. However, since damage and regeneration are very common in natural populations, this kind of study should be restricted to specimens raised in the laboratory and for which the past history is known.

CONCLUSION

This paper is just a preliminary account; here I just tried to expand in a comparative way the detailed study already performed in *A. aquaticus*. However, a conclusion can be obtained from this, the phylogenetic variation in the segmentation process involves only the division(s) of the articles produced by the meristematic one. Further specific studies taking into account more species and with more detailed observation can provide very interesting data for students of evolutionary developmental biology as well as isopod phylogenetic.

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SEGMENTAL MISMATCH IN ARTHROPOD APPENDAGES: THE NAUPLIAR ANTENNAL EXOPOD OF *ARTEMIA* (CRUSTACEA, BRANCHIOPODA, ANOSTRACA)[*]

ABSTRACT

Using traditional techniques as well as a new technique based on the use of a confocal laser scanning microscope for studying external morphology, and immunohistochemical techniques for studying the muscular system, we here describe the *Artemia* naupliar antennal exopod. Two main serial elements are present in the antennal exopod: posterior-ventral setae (with some cuticular folds at their base) and anterior-dorsal cuticular folds (coupled with a row of denticles); these two structures, however, do not exactly match. The folds on the two side of the exopod are also the sites of intermediate insertions of the three muscles of the exopod, and since the two tegumentary structures are discordant, this is reflected also on the pattern of muscle insertions on the two sides. Similar cases of "segmental mismatch" are well known for the trunk of several arthropod taxa; however, cases of segmental mismatch along the appendages have received very little attention. The occurrence of segmental mismatch in the naupliar appendages of both extant and fossils arthropods is briefly reviewed and problems in the interpretation of these data on a phylogenetic framework as well as problems of evolution and development are discussed.

INTRODUCTION

Adult anostracans have sexually dimorphic second antennae. Those of the females are usually small, while those of the males modified into large claspers used to grasp the female during mating (SCHRAM, 1986). However, these divergent morphologies are acquired later in postembryonic development, since during the early postembryonic instars, the naupliar stage, second antennae in both sexes present the usual morphology also found in other branchiopod (and crustacean) nauplii. Crustacean nauplii are defined by having three pairs of functional appendages: first antennae (or antennules), second antennae and mandibles. The naupliar stage is usually divided in two phases of one or more instars each: orthonaupliar, with only the naupliar appendages present, and metanaupliar, with one or more postmandibular appendages present but not yet completely developed and functional. The second antennae of the nauplius are usually biramous, the proximal protopod bearing the outer and usually longer exopod and the inner and usually shorter endopod. Both rami are generally described as "multisegmented" or "multiannulated". This is the case also for the antennal exopod of several anostracan branchiopod nauplii (e.g., FRYER, 1983; MØLLER *et al.*, 2004; OLESEN, 2004). However, from available descriptions of the antennae of *Artemia* nauplii their segmental condition (i.e., the pattern of the division in articles) is unclear. Some authors are silent about the segmentation of the exopod (e.g., HEATH, 1924; COHEN *et al.*, 1999), others explicitly describe the latter as unsegmented (e.g., GAULD, 1959; SCHREHARDT, 1987).

The internal anatomy of naupliar appendages is generally poorly known in crustaceans. A well-described case is the distinctly jointed exopod of the second antennae of cephalocarids: it is divided in articles by well-formed joints (or article

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[*] This paper in advanced status of preparation for eventual submission to *Arthropod Structure & Development* (see p. 26), authors Diego Maruzzo, Alessandro Minelli & Giuseppe Fusco.

boundaries) and does not change its structure from nauplius to adult. It has muscles running parallel to the proximo-distal axis (some of them extending till the last joint), usually having an intermediate insertion on each joint they pass through (HESSLER, 1964). The musculature of the naupliar antennal exopod of *Artemia* has been described only partially (BENESCH, 1969; KIERNAN & HERTZLER, 2006; identical results have been reported by FRYER (1983) for another anostracan, *Branchinecta ferox* (Milne-Edwards, 1840)). Three muscles run throughout the exopod, one in the dorsal and two in the ventral side. None of these works, however, provides detailed information on muscle insertions, although other authors briefly mentioned the existence of intermediate muscle insertions (MACRAE *et al.*, 1991; CRIEL & MACRAE, 2002).

Here we describe the external morphology and the musculature of *Artemia* naupliar antennal exopod. The results are compared with several extant and fossils arthropods, and the comparison is then discussed both under a phylogenetic and a comparative developmental point of view.

MATERIALS AND METHODS

Artemia nauplii freshly hatched from cysts (obtained from INVE, Belgium) were fixed overnight at 4°C in 4% paraformaldehyde in PBS (to study the external morphology with light microscopy and the muscular system) or in 2% paraformaldehyde, 2.5% glutheraldehyde in PBS (for scanning electron microscope observation), washed in PBS and eventually stored in PBS with 0.05% of sodium azide at 4°C. Only orthonaupliar and early metanaupliar stages (less than one day old) were studied.

External morphology was studied with light microscopy, confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). For light microscopy, specimens digested in KOH were washed in PBS with 0.3% Triton-X 100 and mounted in glycerol. Observations were made with a Leica DM5000 B microscope using bright field light or differential interference contrast (DIC). In recent time it has been shown that the external morphology of some arthropod appendages can be studied in great detail with CLSM by taking advantage of the autofluorescence of the cuticle (ZILL *et al.*, 2000; KLAUS *et al.*, 2003; KLAUS & SCHAWAROCH, 2006; MICHELS, 2007). This procedure is also effective for the naupliar appendages studied here, but we obtained better results by staining the digested specimens with Evans Blue; this is a non specific stain that produces a bright red fluorescence in the cuticle of the digested specimens. After digestion, specimens were thus stained in 0.005% Evans Blue in water for 2 minutes, washed three times in water, mounted in glycerol and studied with 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. For SEM observations specimens were dehydrated in graded ethanol series, dried with hexamethyldisilazane (Sigma) (NATION, 1983), and coated with gold. Examinations were made with a Cambridge Stereoscan 260 or with a Jeol JSM-6490 scanning electron microscope.

The musculature was investigated with phalloidin staining (which stains filamentous actin), while the pattern of muscle insertion (which can also be observed with phalloidin staining; see below) was studied with an antibody against α-tubulin (for which *Artemia* muscle insertions are known to be rich; CRIEL *et al.*, 2005). Specimens were stained for actin only or both actin and α-tubulin. For double staining specimens were briefly sonicated (to improve penetration) and incubated in PBS with 0.3% Triton-X 100, 1% BSA, and 2% rabbit serum for 1-2h at room temperature. Primary antibody against α-tubulin made in mouse (Sigma) was used (1:750, overnight at 4°C). After several washes in PBS, samples were incubate for 1h at room temperature in fluorescein-conjugated phalloidin (0.5 μg/ml in PBS; Sigma); they were then washed three more times in PBS and incubated for 4h with rhodamine-conjugated anti-mouse secondary antibody (1:200 at room temperature; Sigma). After several washes (the last one overnight at 4°C), samples were mounted in gel mount aqueous mounting medium (Sigma), and observed with an epifluorescence microscope or with a CLSM. Single staining (with phalloidin only) were performed as above but omitting the incubation with serums and antibodies. Controls treated as described but without both primary antibody and phalloidin resulted in lack of any specific signal, although autofluorescence of both cuticle and internal tissues was present.

The actual location of muscle insertions on the tegument was observed on specimens stained for actin or both actin and tubulin under epifluorescence using also DIC or the blue filter under epifluorescence to visualize the cuticle.

RESULTS

EXTERNAL MORPHOLOGY

The external morphology of the antennal exopod of *Artemia* nauplii is shown in Fig. 4.1. On the posterior-ventral side there are long, natatory setae. These setae (in number of 8-10 plus a small apical one) have a cuticular fold at their base, resembling a joint; in the following we call it "setal cuticular fold". In the opposite anterior-dorsal side there are small joint-like cuticular folds, in the following we call them "anterior-dorsal cuticular folds". Their number is variable (from 8 to 14), and each one of it is accompanied by a row of denticles just proximal to it. Denticles are usually present (although not arranged in rows) also in other part of both the appendages and the body. Variation in the number of anterior-dorsal cuticular folds does not seem to be due to a postembryonic increase since no difference was found between orthonauplii and metanauplii.

While both setae and anterior-dorsal cuticular folds show a clear serial arrangement, the two series are only rarely perfectly matching, usually, some mismatch is obvious (Fig. 4.1).

THE MUSCULAR SYSTEM

As already noted in *Artemia* (BENESCH, 1969; KIERNAN & HERTZLER, 2006) as well as in the only other anostracan studied, *Branchinecta ferox* (see FRYER, 1983), three muscles run throughout the antennal exopod, two on the side of the natatory setae and one on the opposite side. All these muscles have several intermediate insertions along their course (Fig. 4.2). The muscle insertions are easily visualized as thin filaments of both actin (Fig. 4.2) and α-tubulin, which are perfectly overlapping (Fig. 4.3). These filaments (the tendons), that "come out" from the muscle, end in the cuticle, exactly at the setal cuticular folds (for the two muscles on the posterior-ventral side) or in the anterior-dorsal cuticular folds (for the muscle on the other side) (Fig. 4.4), so the mismatch between setae and folds is reflected also in the pattern of muscle insertion (Fig. 4.2 and 4.4). The muscles have their terminal insertions at the setal cuticular folds of the seta proximal to the small terminal one or in the distalmost anterior-dorsal cuticular fold (Fig. 4.4).

DISCUSSION

A segmental pattern may be defined as the serial occurrence of homologous structures along one axis, being that the trunk body axis or the axis of an appendage. However, when this pattern is referred to the whole axis, rather than to

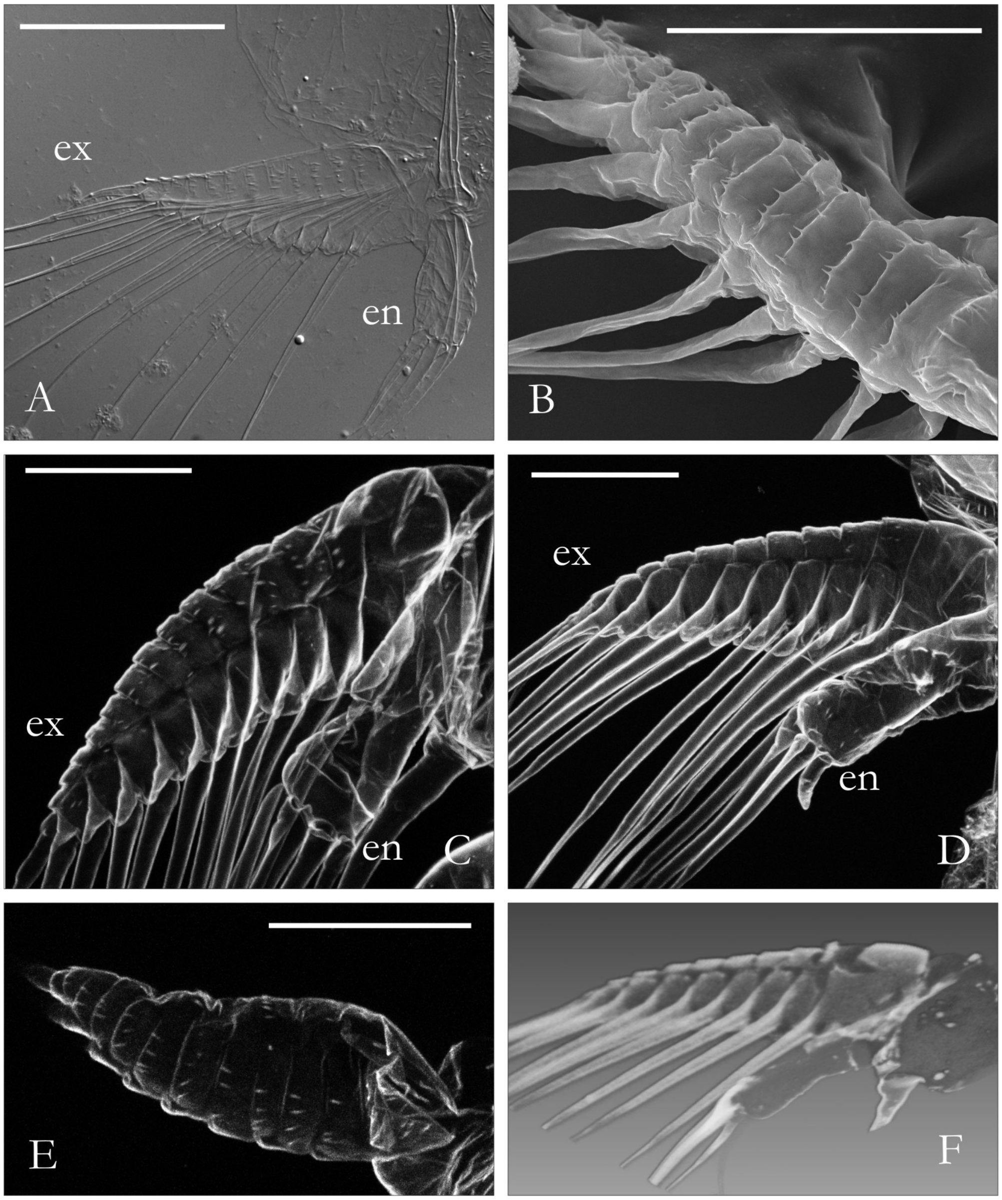

Fig. 4.1. The external morphology of the second antenna of a nauplius of *Artemia*. A, DIC; scale bar 100 µm. B, SEM; scale bar 50 µm. C, D, E, maximum intensity projection of a stack of pictures obtained with CLSM; scale bars 50 µm. In E a projection of selected pictures of a stack to show just the anterior-dorsal cuticular folds with their rows of denticles. F, a volume rendering (made with AmiraDev 4.0) of a stack of pictures obtained with CLSM. Note that the segmental arrangement of the natatory setae, on the posterior-ventral side, do not match with the segmental arrangement of the anterior-dorsal cuticular folds. ex, exopod; en, endopod.

a specific set of repetitive structures, the idea of a trunk or a limb “comprised of” a certain number blocks (trunk segments, appendage articles) will result. This concept of segment, as a unit repeated along an axis, may not reflect the developmental origin of the segmental structures (e.g., JANSSEN *et al.*, 2004; MINELLI, 2004) and descriptive difficulties arise when different serial structures present on the same axis show discordant serial arrangement. In this case we speak of segmental mismatch between different structures along the same axis.

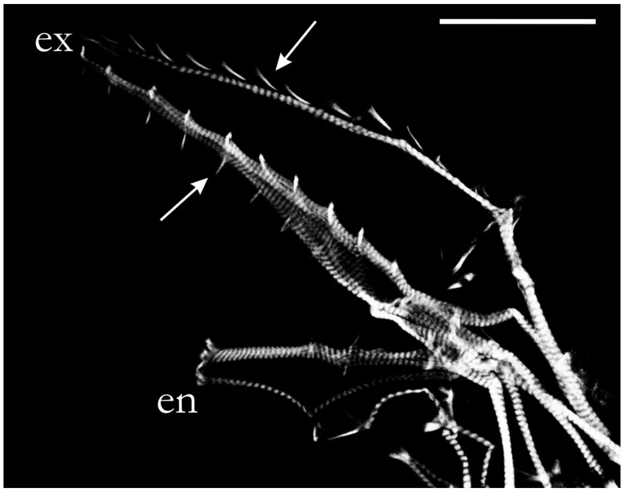

Fig. 4.2. Musculature of the second antenna of a nauplius of *Artemia* (maximum intensity projection of a stack of pictures obtained with CLSM, phalloidin staining). Arrows point to two intermediate muscle insertions (among many). ex, exopod; en, endopod. Scale bar 50 µm.

Cases of segmental mismatch in the trunk of arthropods are not so rare as it may be thought. The most celebrated case is probably that of the notostracan crustaceans, with their marked differences in periodicity, length of the series and postembryonic segmentation schedule among dorsal and ventral structures of the trunk (LINDER, 1952). Very numerous and diverse cases of mismatch in the trunk are also present in myriapods (see FUSCO, 2005). "Spiral segmentation" (segment boundaries with an helicoidal shape; also called helicomerism after BALAZUC & SCHUBART, 1962) is also known in arthropods, although generally associated to a teratological condition; helicomerism have been described for the trunk of insects (e.g., RAMSAY, 1959 and references therein), myriapods (e.g., BALAZUC & SCHUBART, 1962; MINELLI & PASQUAL, 1986) and, among crustaceans, only for notostracans and pentastomids (LINDER, 1947, 1952 and references therein).

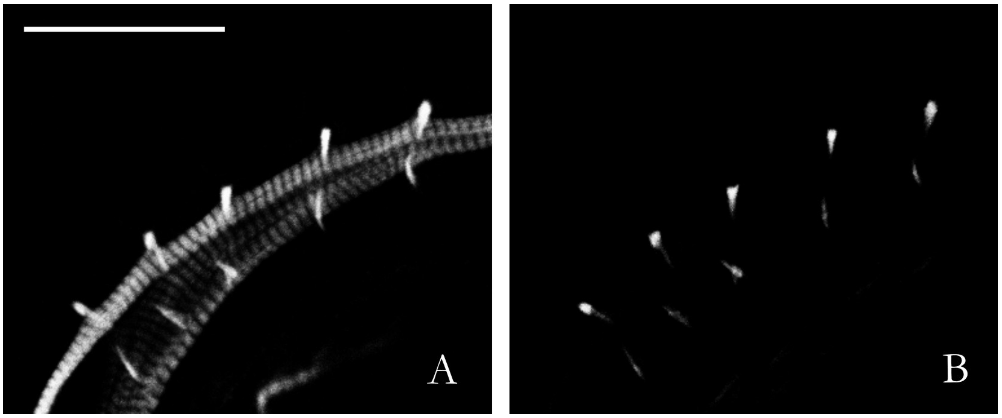

Fig. 4.3. Muscles and their insertions in the antennal exopod of a nauplius of *Artemia* (maximum intensity projection of a stack of pictures obtained with CLSM). The same portion of the appendage is shown to visualize both actin (A; phalloidin staining) and α-tubulin (B; antibody against α-tubulin staining). Scale bar 30 µm.

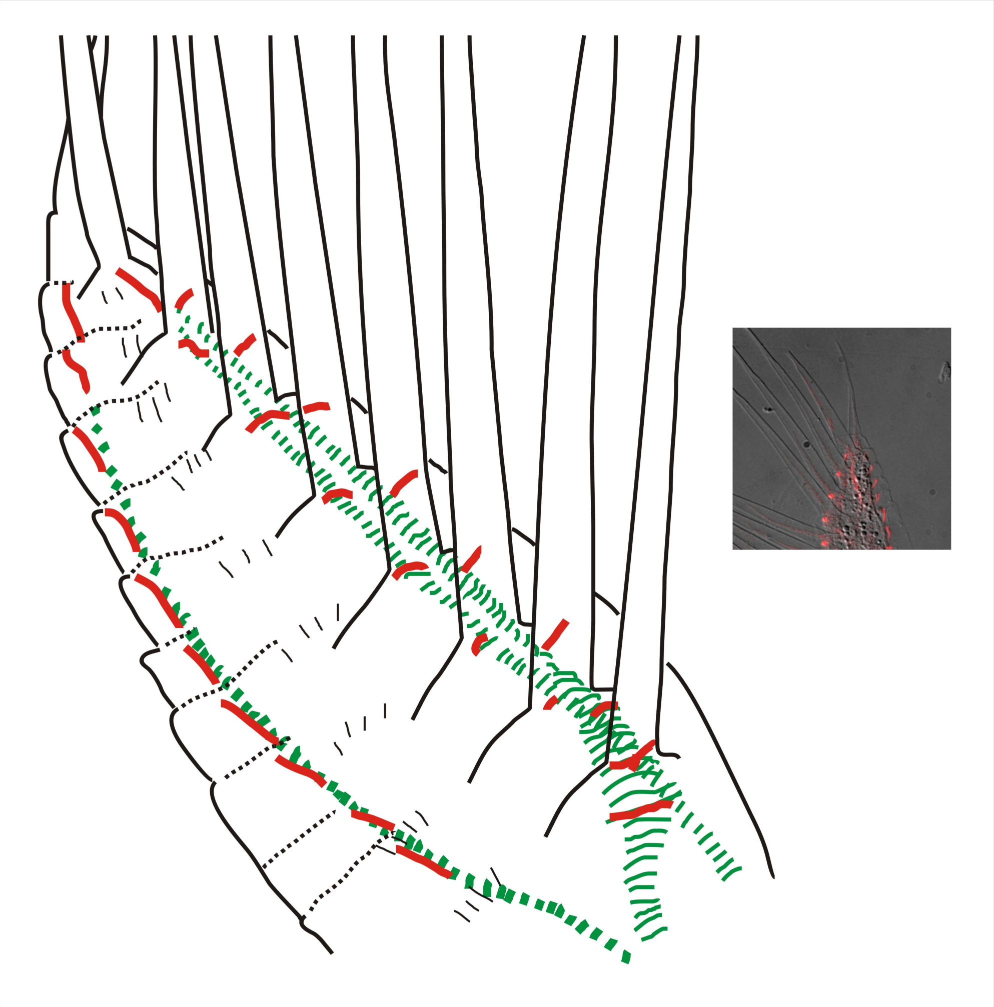

Fig. 4.4. Schematic drawing of the naupliar antennal exopod of *Artemia*. Cuticle is in black, muscles are in green and muscle insertions are in red. The drawing, based on one specimen, was made digitally from different focus-level photographs as described in the materials and methods for studying the location of muscle insertions. Inset shows an overlay of photographs obtained with DIC and red epifluorescence filter (antibody against α-tubulin staining).

Segmental mismatch along the appendages has received very little attention, but it is conspicuous in the naupliar antennal exopod of *Artemia* described in this paper. At the level of external morphology it is possible to identify two segmentally arranged structures in the exopod: setae (with setal cuticular fold) and anterior-dorsal cuticular folds (each coupled with a row of denticles). These segmentally arranged structures, however, do not necessarily match. In the following section I provide a review of similar structures and segmentation mismatches in other crustaceans. While in describing the exopod of *Artemia* naupliar antennae we mainly referred to "cuticular fold", the terminology used by different authors for similar structures in other branchiopods (and in other crustaceans as well as) is far from uniform; thus, irrespectively of the actual terms used by different authors, whenever possible we use in the following the terminology employed here for *Artemia*.

COMPARISON WITHIN CRUSTACEA

The two rami of naupliar second antennae of branchiopods are generally described as "multisegmented" or "multiannulated". For instance, in the nauplii of the anostracan branchiopod *Eubranchipus grubii* (Dybowski, 1860), the appendage presents one seta on each "annulus" (MØLLER *et al.*, 2004; see their Fig. 2D), i.e., the series of setae and the cuticular folds on the opposite side are matching along the exopod. A well-segmented antennal endopod and exopod are also present in the nauplius of the laevicaudatan *Lynceus brachyurus* Müller, 1776 (OLESEN, 2005). However, cases of "ambiguous segmentation" are not rare. The antennal endopod of *E. grubii* bears no setae along most of its length, but only on the tip. The endopod has "unclear segmentation", revealed by "rows of minute spines" (i.e., denticles) and "articulation-like constrictions" (i.e., cuticular folds not producing a complete ring) (MØLLER *et al.*, 2004; see their Fig. 4C and 7C). A comparable morphology is also found in the naupliar antennal endopod of *Artemia* (SCHREHARDT, 1987; see his Fig. 5).

Among other extant branchiopods, spinicaudatans exhibit an interesting feature. During the postembryonic development of *Caenestheriella gifuensis* (Ishikawa, 1895), the segmentation of both the antennal exopod and endopod gets reduced. This reduction, however, does not involve the few setae present along the rami, but only the rows of denticles and their associated cuticular folds (OLESEN & GRYGIER, 2004; see, e.g., their Fig. 8B); thus, the two sides of the appendage show a remarkable degree of independence.

In the Upper Cambrian *Rehbachiella kinnekullensis* Müller, 1983 (a fossil crustacean with branchiopod affinities; e.g., MØLLER *et al.*, 2004; OLESEN, 2004), the proximal part of the first antenna is made of "incomplete annuli": cuticular folds, whose number increases slightly during postembryonic development, are present only on the anterior side (thus not producing a complete ring), with one row of denticles slightly proximal to them and segmentation indistinct on the posterior side (WALOSSEK, 1993). In the exopod of both second antennae and mandibles the setae (on the posterior side) may (WALOSSEK, 1993; see Fig. 3 of his plate 19 for an antennal exopod) or may not (WALOSSEK, 1993; see Figs. 3 and 4 of his plate 4 for an antennal exopod and Fig. 2 of his plate 9 for a mandibular exopod) match with the cuticular folds (which are always equipped with a row of denticles) on the anterior side. Concordance or mismatch between the anterior cuticular folds and posterior setae of these appendages was also one feature used by WALOSSEK (1993) to distinguish two possible alternative ontogenetic pathways ("larval series") in *R. kinnekullensis*.

"Several partial rings of minute teeth" (i.e., cuticular folds with rows of denticles not making a complete ring) have been also described (SCOURFIELD, 1940) for the larval stage of the Devonian *Lepidocaris rhyniensis* Scourfield, 1926 (possibly, a stem lineage anostracan; MØLLER *et al.*, 2004).

In the Upper Cambrian *Bredocaris admirabilis* Müller, 1983 (a fossil crustacean with possible thecostracan affinities; e.g., MÜLLER & WALOSSEK, 1988) the first antennae are composed of three articles, but the most proximal one, which is much longer than the others, is further divided into short articles (apparently, the actual number increased slightly during postembryonic development) at the anterior side only. These "articles" are cuticular folds, limited to the anterior side, with the usual row of denticles slightly proximal to them (MÜLLER & WALOSSEK, 1988). The exopod of the second antennae has "ring-like segments" made of cuticular folds with their row of denticles on the outer side only. On the inner side there are the natatory setae (actual number with individual variation) which does not exactly match with the cuticular folds on the opposite side of the appendage (MÜLLER & WALOSSEK, 1988; see especially Fig. 6 in their plate 8, and Fig. 5 in their plate 15). A similar situation is also found in the mandibular exopod (MÜLLER & WALOSSEK, 1988; see especially Fig. 6 of their plate 4).

In the Upper Cambrian *Skara* (a fossil crustacean with unclear affinities) the segmentation of the exopod of both second antennae and mandibles is not always regular, since "on the posterior side there are more joints than on the anterior one" (MÜLLER & WALOSSEK, 1985; see Fig. 4 and 8 of their plate 7). In this case, however, the mismatch between posterior and anterior side is not due to a different number or position of serial structures on different sides, but is apparently due to a kind of "spiralling" of the joints, i.e., starting from the base of a seta the joint proceeds as an helix and do not exactly meet on the opposite side. While MÜLLER & WALOSSEK (1985) did not describe any mismatch in the first antenna, their Fig. 7A shows some mismatch as different number and position of serial structures between the opposite sides of the appendage.

In the extant cirripede *Briarosaccus tenellus* Boschma, 1970, the joints in the naupliar exopod of both second antennae and mandibles are not complete rings; as in *Skara*, this seems to correspond to a "spiralling" joint (WALOSSEK *et al*., 1996; see their Figs. 8C, 12G and 21C). Identical morphology has also been found in the naupliar exopod of second antennae and mandibles of another cirripede, *Balanus improvisus* Darwin, 1854 (personal observations).

It is thus clear that the segmental mismatch described here for *Artemia* is not an isolated case. At the present, however, it is not possible to clearly understand if these mismatch are independently evolved feature or can be trace back to a common ancestor. This is because, while for other crustacean nauplii such as those of cephalocarids or copepods, similar mismatch have not been recorded, these fine morphological details may have been well overlooked in taxa where they have not been explicitly looked for.

DIFFERENT KINDS OF SEGMENTAL MISMATCH?

WALOSSEK *et al*. (1996) considered the peculiar "annulated design" of *Briarosaccus* and *Skara* identical to that present in *Rehbachiella* and *Bredocaris* (which seems very similar to the one shown here in *Artemia*). In *Briarosaccus* (and also in *Balanus improvisus*; personal observations) the mismatch of serial features along both the antennal and mandibular exopod seems to be produced by the cuticular folds of the joints which are aligned along the proximo-distal axis on the same side as the setae, but have a "spiral course" and do not meet on the opposite side, so they do not produce complete rings; this morphology clearly resemble the helicomerism (spiral segmentation) described for the trunk of different arthropods in teratological cases (see above). Also the antennal and mandibular exopod of *Skara* seem to have this morphology. The condition of *Rehbachiella*, *Bredocaris* and *Artemia* seems different. In these cases the mismatch is due to a relative independence of the opposite sides, where different serial structures have different number and position.

While the existence of two different kinds of segmental mismatch seems more probable, caution should be used. Fig. 1 of plate 11 in WALOSSEK (1993) (a picture not mentioned by WALOSSEK when discussing the exopodal mismatch) shows the antennal exopod of a postlarval stage of *Rehbachiella* with "spiralling" cuticular folds. It must be pointed out that a link between incomplete segments and spiral segmentation in the trunk of notostracan crustaceans has already been proposed (LINDER, 1947). In addition, while the antennal and mandibular exopod of *Skara* seems to show helicomerism, the first antenna seems to have the typical mismatch as different number and position of serial structures between the opposite sides.

To complicate things further, the spiralling joints of cirripedes end, on the opposite side of the setae, on a longitudinal fold that goes through all articles, parallel to the proximo-distal axis. This fold appears very similar to the groove or channel observed by MÜLLER (1979) in some species of Upper Cambrian phosphatocopines (now considered the sister group of Eucrustacea [a group containing all extant crustacean taxa]; see, e.g., MAAS & WALOSZEK, 2005), although no segmental mismatch has been noted in the appendages of this group. At the present, many questions concerning the homology of the described structures are open. Further studied are needed.

ON SETAE, CUTICULAR FOLDS AND JOINTS

According to MØLLER *et al.* (2007) one seta per article is the primitive condition for the antennal naupliar exopod of crustaceans; thus, they considered the distally placed setae arranged in a "step-like" manner in the larval antennal exopod of the branchiuran *Argulus foliaceus* (Linnaeus, 1758) as a form of "effaced segmentation". In *Artemia*, SCHREHARDT (1987) described the rows of denticles in the naupliar antennal exopod (he did not describe the anterior-dorsal cuticular folds) and, while considering the exopod unsegmented, interpreted them as vestigial articulation. Also, cuticular folds have been considered as "signs of segmentation" in naupliar appendages in several papers dealing with branchiopod postembryonic development (e.g., OLESEN & GRYGIER, 2003, 2004; MØLLER *et al.*, 2004; PABST & RICHTER, 2004). So, both setae (which always have some cuticular folds at their base) and cuticular folds (not producing a complete ring around the appendage) have been interpreted as forms of "reduced" segmentation.

If we accept that the primitive condition was a well segmented appendage (an appendage divided in articles by well-formed joints), a legitimate question is: are a set of matching anterior-dorsal cuticular folds and setae (with setal cuticular folds) really homologous to a complete joint? Or, alternatively: are the anterior-dorsal cuticular folds or the setae (with setal cuticular folds) an evolutionary novelty and is the other serial structure a vestigial sign of "reduced segmentation"? We accept here that a set of matching anterior-dorsal cuticular folds and setae (with setal cuticular folds) are homologous to a complete joint and are the primitive condition. This is because one seta per article is found in the nauplii of many extant crustaceans as well as in phosphatocopines (see, e.g., SIVETER *et al.*, 2003) which according to MAAS & WALOSZEK (2005) are the sister group to Eucrustacea. Additional evidence is provided by the muscular system (see below).

In the naupliar appendages of panbranchiopods (branchiopods plus *Rehbachiella*), the series of setae and cuticular folds on the opposite sides show different relationships (see previous references): 1) good matching (e.g., antennal and mandibular exopod of *Rehbachiella* [in one larval series], antennal exopod of *Eubranchipus*, antennal endopod and exopod in the levicaudatan *Lynceus brachyurus*); 2) mismatch (e.g., antennal and mandibular exopod of *Rehbachiella* [in one larval series], antennal exopod of *Artemia*); 3) presence of cuticular folds (not producing a complete ring) only (e.g., first antennae of *Rehbachiella* and *Lepidocaris*, antennal endopod of *Eubranchipus* and *Artemia*). In addition, in the antennal exopod and endopod of *Caenestheriella*, postembryonic development involves changes only in the cuticular folds, not in the setae. All these examples show that setae and cuticular folds of panbranchiopods, although very likely derived from a complete joint, can now behave as independent segmental units.

CUTICULAR FOLDS AND MUSCLE INSERTIONS

More or less extensive sections of many crustacean appendages have only muscles that run parallel to the proximo-distal axis with intermediate muscle insertions on each joint they pass through (e.g., first antennae, exopods of the second antennae, exopods of naupliar mandibles, endopod of first and second maxillae and of thoracopods of cephalocarids, HESSLER, 1964; first antennae of copepods, BOXSHALL, 1985; cirri of cirripedes, STUBBINGS, 1975). This is also somehow true for the antennal exopod of *Artemia* nauplii: both the setal cuticular folds and the anterior-dorsal cuticular folds provide sites for (intermediate) muscle insertions; since the setal cuticular folds and the anterior-dorsal cuticular folds are not serially arranged concordantly, this is reflected also in the pattern of muscle insertion on the opposite sides (Fig. 4.2 and 4.4). This is also an evidence that well-correlated setal cuticular folds and anterior-dorsal cuticular folds are homologous to joint: if setal cuticular folds and anterior-dorsal cuticular folds derived from a complete ring of a joint which posses intermediate insertion(s) of muscle(s) running parallel to the proximo-distal axis, this is what one would expected.

The evolution of the developmental relationship between muscle insertions and cuticular folds (that if they produced a complete ring are called joints, article boundaries or segment boundaries) are far from clear, and specific studies for appendages are lacking indeed (see WILLIAMS & NAGY, 1996 and BUDD, 2001 for discussion on appendage and trunk segmentation, respectively). In insect legs, development of tendons of the joints and muscles are very closely correlated (BALL *et al*., 1985) and the microsurgical suppression of the development of the tendons interferes with the differentiation and segregation of the muscles that would insert there (FOURNIER, 1968); in addition, the molecular mechanism for the development of the joints and muscle insertions is partially similar (SOLER *et al*., 2004). In the model we studied the appendage cannot be subdivided in articles since cuticular folds do not produce complete rings, but a close developmental relationship between cuticular folds and muscle insertions appears to be present. However, a close developmental relationship between muscle insertion and joint is far from universal since joints without muscle insertions are rather common in arthropod appendages (reviewed in BOXSHALL, 2004).

CONCLUSION

When dealing with the process of segmentation in arthropod appendages, the attitude is to consider it just a problem of positional information along the proximo-distal axis. We do not question this, but things may be more complex than what expected. We have shown that the positional information for structures likely homologous to parts of joint located on different sides along the proximo-distal axis may not be used in the same way.

Segmental mismatch is well known for the trunk of different arthropods, but has received very little attention in the case of the appendages. The case of *Artemia* naupliar antennae described here, along with all the other cases in extant and fossil arthropods summarized in the previous pages demonstrate the existence of this phenomenon and that its occurrence could have been overlooked. It is, however, premature to make evolutionary interpretation because the actual homology of the described cases needs more specific studies and because the absence of mismatch in other species may well be due to a lack of specific studies.

Setae and cuticular folds on the opposite side (likely ancestrally united in a complete ring, a joint) may be rather independent, and many examples of it are available for branchiopods. If the ancestral condition was a well-segmented appendages with muscles running parallel to the proximo-distal axis and having intermediate insertions on each article boundary they pass, than the developmental relationship between muscle insertion and cuticular fold has here been maintained.

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ARTICLE NUMBER AND MUSCULAR SYSTEM IN THE NAUPLIAR APPENDAGES

ABSTRACT

This paper reviews knowledge on the ontogenetic variation in article number and structure of the muscular system in the naupliar appendages. Since knowledge on the muscular system is usually scarce, and since in some groups these appendages undergo profound modifications, the musculature of the naupliar appendages of a representative of one of these groups (the cirriped *Balanus improvisus*) is also described. These data on naupliar appendages are used to discuss possible differences between true articles and annuli in arthropod appendages, as suggested in previous works. It is concluded that there is no evidence that any consistent developmental difference between true articles and annuli can be generalized to different appendages and different groups, and that the developmental differences between these two kind of articles (which, however, need to be redefined) simply reflect their different functional morphology.

INTRODUCTION

Two main kinds of articles (or segments) are usually recognised in arthropod appendages: “true” articles and annuli. The distinction among them is usually claimed to be based on the underlying muscular system: true articles are equipped with intrinsic muscle(s), while annuli are not (SNODGRASS, 1935; BOXSHALL, 2004). Students of chelicerates (e.g., SHULTZ, 1989; VAN DER HAMMEN, 1989), following COUZIJN (1976), usually prefer to refer to eudesmatic and adesmatic joints (or article boundaries), with the former defined as joints where at least one muscle insert, and the latter with no muscle insertion. Indeed, the process of “annulation” (production of annuli) is often conceived as a different process, if compared to “segmentation” (production of true articles) and annuli are often conceived just as secondary subdivisions of articles (e.g., SNODGRASS, 1935; MCLAUGHLIN, 1982; SHULTZ, 1989; BOXSHALL, 2004).

Studies from the fruit fly also provide evidence that the mechanism of annulation is different from the production of true articles. In *Drosophila* appendages the only articles that can be classified as annuli are the distalmost ones of the legs, the tarsomeres. The molecular mechanism involved in tarsus patterning has peculiar differences in respect to the remaining leg patterning (reviewed in KOJIMA, 2004). Also, removing homeotic-selector gene influence from leg development causes the production of a leg with all articles fused together, but for the tarsomeres, which are normally produced (CASARES & MANN, 2001). At the cellular level, while the cell shape changes involved in joint production are basically the same between tarsomeres and other articles (MIRTH & AKAM, 2002), recent findings show that apoptosis is required for proper formation of tarsomeres only (MANJÓN *et al.*, 2007).

Based on comparative data on arthropod appendage morphology and development, BOXSHALL (2004) was the first to suggest that the timing of development is important for the production of true articles and annuli. Annuli tend to appear relatively later in development than true articles. A similar observation was also made for arthropod appendage regeneration where “the number of flagellar units [annuli] in the regenerate increases from moult to moult, whereas all ‘true segments’ [true articles] are usually formed as soon as the regenerate comes out first" (MARUZZO *et al.*, 2005: 236). MARUZZO *et al.* (2007) discussed further this ontogenetic difference. They noted that most true articles are produced in particular developmental periods (such as embryogenesis or metamorphosis), while annuli do not necessarily do so; new annuli can continuously be added in some sections of appendages during postembryonic development. Anyway, they also noted that this is not valid for all true articles. Indeed, true articles in which their intrinsic muscle insertions are intermediate insertions of a muscle (i.e., the muscle does not originate or end there) can develop more like annuli than like true articles.

To elucidate better these developmental particularities of arthropod appendage articles, and their evolutionary implications, three aspects need to be considered in a comparative way: a) the ontogenetic variations in the number of articles; b) the anatomy of the muscular system; c) the actual muscle insertion ([proximal] origin, intermediate insertion(s), and terminal [distal] insertion). However, data on all these three different aspects in appendages of different arthropods to be used for comparative purpose are often lacking. From the literature it is possible to obtain data on article number (even if not always of comparable quality), while descriptions of appendage intrinsic musculature are much more limited, especially for larval stages, and, among these papers, precise accounts on muscular insertion are almost a rarity.

I provide here a review on the ontogenetic variations in article number and on the structure of the muscular system in naupliar appendages. The naupliar is the first (and sometimes the only) larval phase of many different crustaceans (cf. DAHMS *et al.*, 2006; a review to be updated with the recent discover of remipede nauplii, KOENEMANN *et al.*, 2007). Crustacean nauplii are defined by having three pairs of functional appendages (first antennae [or antennule], second antennae and mandibles; in the following simply called naupliar appendages). This phase is usually divided into orthonauplius (only naupliar appendages present) and metanauplius (one or more postmandibular appendages present externally, but not yet completely developed and functional).

The naupliar appendages are a useful model for our purpose, because nauplii are usually more similar in morphology than the corresponding adults. Generally, the first antennae are uniramous, while both the second antennae and the mandibles, which are usually similar to each other, are biramous and composed of a proximal part (protopod) which bears two rami, the exopod and the endopod. The exopod is usually the longer and is usually described as multisegmented or multiannulated. In the following I consider mainly the exopod of the biramous naupliar appendages because this element shows more variation in article number, both ontogenetical and phylogenetical. The protopod is one- or two-segmented (see, e.g., MCLAUGHLIN, 1982; BOXSHALL, 2004; FERRARI & DAHMS, 2007 for a discussion on the occurrence of a three-segmented protopod in other arthropod appendages). The endopod has usually fewer articles than the exopod and it is often modified. Some considerations on the naupliar appendages (first antennae, second antennae and mandibles) will be also done on post-naupliar developmental phases, when appropriate.

While many description of external morphology of naupliar appendages are available, basically covering all major crustacean groups, the knowledge of muscular system is usually poor. In some groups the adult structure of the naupliar appendages is rather similar to the naupliar one. Therefore, if knowledge of internal anatomy is available for the adult, tentative inferences can be made for the nauplius. In other groups, the naupliar appendages go through deeper modifications, so any knowledge on adult internal anatomy is useless in this respect. Among the crustacean groups where this happens are the cirripeds.

In cirripeds, the naupliar phase is followed by another larval phase, the cyprid (e.g., HØEG & MØLLER, 2006). In the cyprids the naupliar appendages are highly modified (first antennae), or almost completely reduced (second antennae and mandibles). Some works deal with cirriped naupliar muscular system (WALLEY, 1969; ANDERSON, 1987; SEMMLER *et al.*, 2006), but none of these provide information on the intrinsic musculature of the appendages. Thus, I provide here a description of the musculature and muscle insertion in the first antennae and in the exopod of both second antennae and mandibles in the nauplii of the cirriped *Balanus improvisus* Darwin, 1854.

MATERIALS AND METHODS

Balanus nauplii (fixed in 4% paraformaldehyde in PBS for 1h at room temperature, washed in PBS and stored in PBS plus sodium azide at 4°C) were kindly provided by Henrike Semmler and were collected as previously described (SEMMLER *et al.*, 2006). Nauplii of *B. improvisus* were staged according to JONES & CRISP (1954).

The external morphology was studied with bright field microscopy or differential interference contrast (DIC). The musculature was studied through phalloidin (for filamentous actin, i.e., muscles) and antibody against α-tubulin (for muscle insertions) staining; single and double staining were made as described in MARUZZO (2008a). Briefly, for improving penetration the bigger specimens of *Balanus* were dissected with fine needles, while the smallest were just briefly sonicated. Blocking solution used was a mix of 0.3% Triton-X 100, 1% BSA, and 2% rabbit serum in PBS; primary antibody against α-tubulin made in mouse (Sigma) was used at 1:750 (overnight at 4°C), secondary antibody anti-mouse rhodamine-conjugated (Sigma) was used at 1:200 (4 hours), fluorescein-conjugated phalloidin (Sigma) was used at 0.5 µg/ml (1 hour); all solutions were made in PBS. Specimens were mounted in gel mount aqueous mounting medium (Sigma), and observed under epifluorescence (with a Leica DM5000 B) or with a confocal laser scanning microscope (CLSM; a Nikon Eclipse E600 microscope equipped with a Bio-Rad MRC 1024ES confocal laser scanning unit was used). Controls treated as described but without both primary antibody and phalloidin resulted in the lack of any specific signal, although autofluorescence of both cuticle and internal tissue was present.

RESULTS

While muscles can be easily visualized through phalloidin staining, muscle insertions can be observed with both phalloidin and antibody against α-tubulin staining (Fig. 5.1). With phalloidin staining muscle insertions appear as “brighter spots” inside a muscle (Fig. 5.1B), while with antibody against α-tubulin staining, although sometimes the nervous system gets also stained, the signal is usually rather specific on muscle insertions (Fig. 5.1C).

It was already known that the first antennae of *B. improvisus* have four articles throughout the naupliar phase (BUCHHOLZ, 1951). There are three muscles running throughout the appendage, inserting in each joint and having the terminal insertion in the last joint. Three more muscles are confined within the two proximal articles, with terminal insertions at the distal joint of the second article (Fig. 5.2A, B). All these muscles run parallel to the proximo-distal axis, with one exception, one muscle of the two proximal articles which, distal to its intermediate insertion at the joint between article one and two, "turns" a little oblique. These muscles are present throughout the naupliar phase (in the following cyprid phase, the first antennae get modified and the musculature deeply changes accordingly; see LAGERSSON & HØEG, 2002 for the musculature of the first antennae of the cyprid of *Balanus amphitrite* Darwin, 1854).

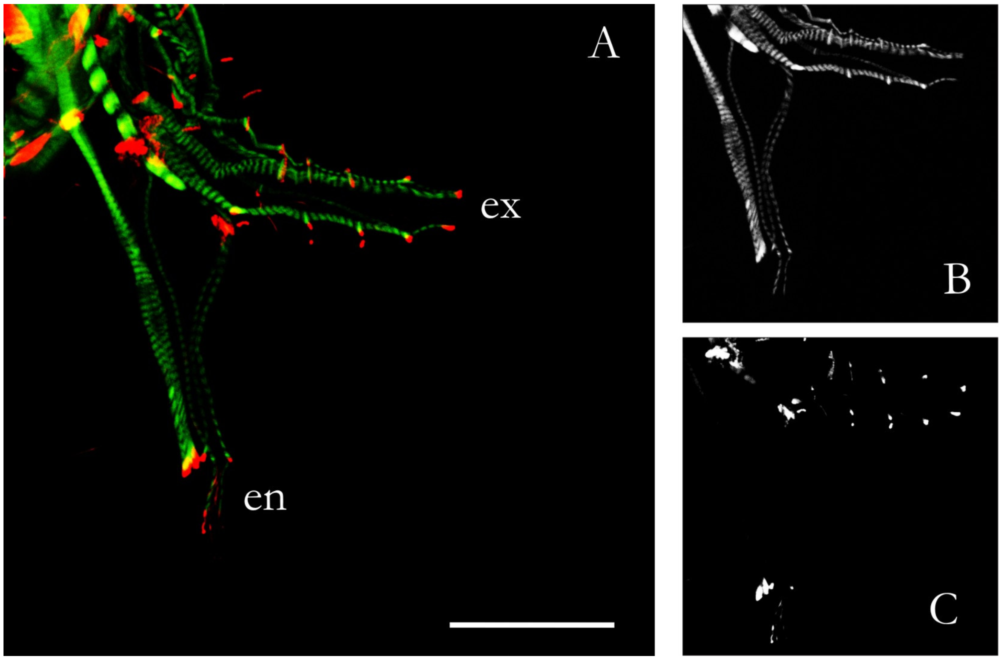

Fig. 5.1. Muscle insertions in a mandible of *Balanus improvisus* sixth stage nauplius. A, merge of two maximum intensity projections obtained with CLSM of a sample stained with both phalloidin (green) and antibody against α-tubulin (red); ex, exopod; en, endopod; scale bar 50 µm. In B and C the original maximum intensity projections of the phalloidin (B) and antibody against α-tubulin (C) signals are shown; B and C not at the same scale as A.

The musculature of the exopod is composed of several muscles that run throughout the exopod, insert on each joint, and have the terminal insertion in the last joint. Few muscles have the terminal insertion in the joints of the proximal part of the exopod (Fig. 5.2C, D). This musculature is constant throughout the naupliar phase. KADO & HIRANO (1994) already shown that the antennal exopod of *B. improvisus* increases in article number during naupliar development, and new joints produced during this phase obtain new intermediate muscle insertions of already present muscles.

In the exopod of the mandibles of *B. improvisus* there are four muscles that run throughout the exopod, insert on each joint, and have terminal insertion at the last joint. Two muscles have the terminal insertion at the joints of the proximal part of the exopod (Fig. 5.1, 5.2). The number of the exopod articles increases from 4 to 5 during the naupliar phase and the new joint is equipped with intermediate muscle insertions of previously present muscles.

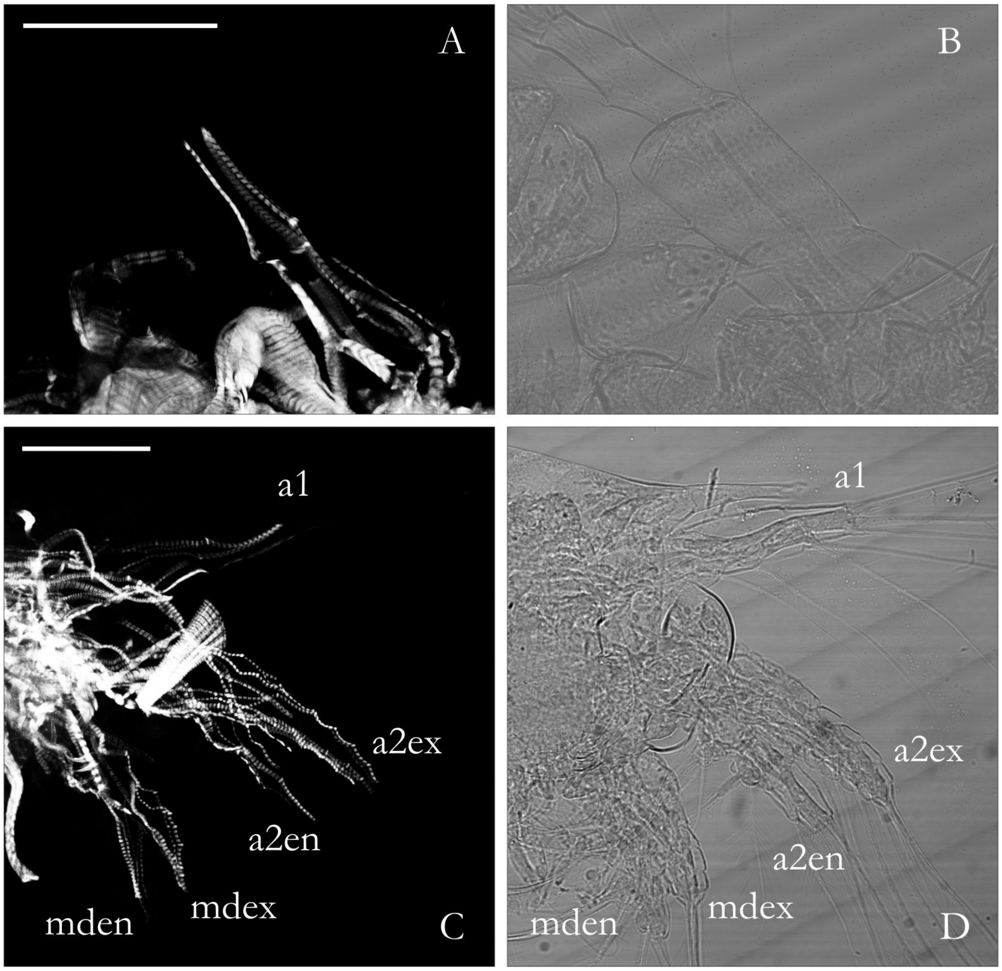

Fig. 5.2. Muscular system of the naupliar appendages of *Balanus improvisus*. A and B, first antenna of a sixth stage nauplius: A, phalloidin staining (maximum intensity projection obtained with CLSM); B, bright field equivalent of A; scale bar 50 µm. C and D, on side of a nauplius of first stage; first antenna (a1), exopod (a2ex) and endopod (a2en) of the second antenna and exopod (mdex) and endopod (mden) of the mandible are shown with both phalloidin staining (C, maximum intensity projection obtained with CLSM) and in bright field (D). Scale bars (B the same of A and D the same of C) 50 µm.

DISCUSSION

ARTICLES AND MUSCULATURE IN THE NAUPLIAR APPENDAGES. A REVIEW

First antennae. Cephalocarida. The muscular system of the first antennae of *Hutchinsoniella macracantha* Sanders, 1955 was described in the adult by HESSLER (1964). The first antennae have many intrinsic muscles, all running parallel to the proximo-distal axis, but inserting in different portion of the appendage. Few muscles are limited to the distalmost part and no muscles are confined within a single article. All joints are equipped with the insertions of some muscles (only possible exception is the joint between the 4th and the 5th article, but this is not clear). The distalmost site of muscle insertion is the distalmost joint. HESSLER (1964) also made observations on late stage larvae, and did not mention any difference for the musculature of the first antennae. In the cephalocarids the first antennae are always composed of 6 articles, from the first naupliar phase to the adult (SANDERS, 1963; SANDERS & HESSLER, 1964; ADDIS *et al.*, 2007).

First antennae. Branchiopoda. The musculature of the naupliar first antennae has been described only in anostracans (BENESCH, 1969; FRYER, 1983; KIERNAN & HERTZLER, 2006) wherethree (*Branchinecta*) or four (*Artemia*) extrinsic muscles end at the base of the appendage. Since, except for laevicaudatans and cladocerans (see below), the structure is very conserved and since the musculature of the first antenna of adult *Caenestheriella australis* (Loven, 1847) (a spinicaudatan branchiopod that as adult has a relatively long first antenna) is, according to BOXSHALL (2004), also very similar, I expect that a condition similar to the anostracan one is generally valid for branchiopods. Branchiopod naupliar first antennae are usually unsegmented (OLESEN, 2004), although some "faint subdivisions" have been noted (see discussion in WALOSSEK, 1993; but see also MARUZZO, 2008a for a deeper discussion on the segmental arrangement of the first antennae of some branchiopods). More particular conditions are found only in laevicaudatans, where the naupliar first antenna is modified into a large anterolateral "horn" (OLESEN, 2004, 2005) and in cladocerans that usually have well-segmented first antennae but also have direct development.

First antennae. *Ostracoda*. An account on the postembryonic changes of the musculature of the first antenna is only available for *Heterocypris incongruens* (Ramdohr, 1808) (see SMITH & TSUKAGOSHI, 2005); unfortunately, the technique employed to study the muscular system (bright field observation on intact first antennae) is inadequate for recognising smaller muscles (indeed, the authors were not able to described the muscular system of the first postembryonic stage) and, especially, for making detailed observations on (intermediate) muscle insertions. Most of the muscles span over more than one article and run parallel to the proximo-distal axis. The first antennae of podocopan ostracods usually acquire new articles during postembryonic development (see SMITH & TSUKAGOSHI, 2005; and references therein), and every new joint produced during postembryonic development can be interpreted as coupled with a new intermediate muscle insertion(s), although new muscles are apparently also produced during postembryonic development.

First antennae. *Thecostraca.* The present account on the musculature of the first antennae of *B. improvisus* is the first for a nauplius of thecostracans (cirripeds plus ascothoracidans and facetotectans). I have shown here that antennal muscles mainly run through the appendage, parallel to the proximo-distal axis; some of them have terminal insertion at the last joint, and insert on each joints they pass through.

The variability in article number and the comparative morphology of the naupliar first antennae of thecostracans have been studied in detail by GRYGIER (1987, 1994), although he did not considered two (rhizocephalan and acrothoracican) of the three (rhizocephalan, acrothoracican and thoracican) major cirriped groups. In the following account I consider separately facetotectan, ascothoracid, rhizocephalan, acrothoracic and thoracic thecostracans.

For facetotectans GRYGIER (1994) reported a 2-segmented condition persisting in all naupliar stages, although evidence for the existence of 3-segmented antennae was discussed. Indeed, also some figures in BELMONTE (2005) seem to support a 3-segmented condition, while the description of KOLBASOV & HØEG (2003) clearly shows a division into two portions, but with the proximal one having circular cuticular folds somehow resembling the proximal annulations seen in the first antennae of some Cambrian crustaceans and extant malacostracan larvae. Anyway, none of the above mentioned papers noted ontogenetic variation.

Ascothoracid nauplii exhibit a maximum of 6 articles, with some division, but also some fusion of articles, common during ontogeny (GRYGIER, 1994).

Acrothoracic cirripeds seem to maintain a 4-segmented status throughout the naupliar phase, although there may be fusion of two articles balanced by the division of one article (TURQUIER, 1967).

Rhizocephalan cirripeds have 3-segmented first antennae during all naupliar stages (WALOSSEK *et al.*, 1996; RYBACOV *et al.*, 2002).

The literature concerning larval development of thoracic cirripeds is huge, but little attention has been paid to the segmentation of the naupliar appendages. Following the initial definition of the setation formula by BASSINDALE (1936), cirripedologists have described in detail the setation of the naupliar appendages, and its modification in different stages (reviewed in NEWMAN & ROSS, 2001), but the number of articles in the naupliar appendages received relatively little attention. In the following reference is made, whenever possible, to papers including clear statements of article number in the text, but to give strength to the conclusions, some papers with just clear figures are also included.

During naupliar development of thoracic cirripeds first antennae, the number of article remains often constant (usually 4; GRYGIER, 1994). This is also deduced from the clear figures of, e.g., BUCHHOLTZ (1951 on *Balanus improvisus*; observation also confirmed in the present study), COSTLOW & BOOKHOUT (1957 on *Balanus eburneus* Gould, 1841, and 1958 on *Balanus amphitrite*), EGAN & ANDERSON (1987 on *Austromegabalanus nigrescens* (Lamarck, 1818)), ANDERSON (1987 on *Ibla quadrivalvis* (Cuvier, 1871)), and KADO & HIRANO (1994 on *Megabalanus volcano* (Pilsbry, 1916) and *Megabalanus rosa* (Pilsbry, 1916)). In some cases, however, an increase has been explicitly noted (MOYSE, 1987 on *Lepas anatifera* Linnaeus, 1758), while in other it is evident from the figures (LANG, 1976 on *Octolasmis mülleri* (Coker, 1902); DINEEN, 1987 on *Lithotrya dorsalis* (Ellis & Solander, 1786)). A combination of fusion and division of articles is sometimes apparent from the figures, although never explicitly mentioned in the text. This could be due to careless figures; however, I noted this among thoracican only for species belonging to the family Chthamalidae (EGAN & ANDERSON, 1989 for *Chamaesipho tasmanica* Foster and Anderson, 1986 and *Chthamalus antennatus* Darwin, 1854; YAN, 2003 for *Chinochthamalus scutelliformis* (Darwin, 1854); YAN & CHAN, 2004 for *Chthamalus neglectus* Yan and Chan, 2004). I then accept this here as a real phenomenon.

First antennae. Mystacocarida. Nothing is known on the muscular system of the appendages of mystacocarids. The first antennae have eight articles from hatching to adult (OLESEN, 2001).

First antennae. *Copepoda.* In adult calanoids, the intrinsic muscular system of the first antenna is composed of muscles originating in the first article and extending until the last joint but having intermediate insertions on each joint they pass through (BOXSHALL, 1985). During the naupliar phase the first antennae of copepods are usually three-segmented, although few species reduce their article number during this phase (FERRARI & DAHMS, 2007). In the following copepodid phase there is usually an increase, sometimes also high, in article number and some fusion of articles may also occur (FERRARI & DAHMS, 2007). BOXSHALL (2004) proposed that the formation of new joints during the copepodid phase happens together with the formation of new intermediate insertions of already present muscles.

First antennae. *Remipedia.* The musculature of adult first antennae, which are biramous, was described by BOXSHALL (2004); while each article of the dorsal ramus has three intrinsic, antagonist muscles, the ventral ramus is completely devoid of muscles. Very little is still known about the postembryonic development of remipedes; however, in the naupliar phase the first antennae are uniramous and unsegmented (KOENEMANN *et al.*, 2007).

First antennae. *Malacostraca.* Among malacostracans, only Euphausiacea and Dendrobranchiata have a naupliar phase, and this is likely a secondarily evolved feature (SCHOLTZ, 2000). There are several accounts on the musculature of adult first antennae, which usually have a proximal peduncle, with articles equipped with intrinsic, antagonist muscles, and one or more flagella devoid of muscles. The structure of the naupliar first antenna is very different from the adult one, and the naupliar muscular system has been described in only one species, *Sicyonia ingentis* (Burkenroad, 1938). While there are six muscles that goes into the naupliar first antenna apparently parallel to its proximo-distal axis, muscle insertions and segmentation of this appendage have not been described (KIERNAN & HERTZLER, 2006). The segmentation of the naupliar first antennae of malacostracans deserves further investigation. These appendages have been described as unsegmented or with indistinct segmentation throughout the naupliar phase in some species (e.g., RONQUILLO *et al.*, 2006) but in other accounts they were described as such only in the first naupliar stages, in later naupliar stages the segmentation is distinct and involved in both acquiring and loosing articles (e.g., HEEGAARD, 1953; FIELDER *et al.*, 1975).

Second antennae. *Cephalocarida.* In the adult exopod, several muscles run parallel to the proximo-distal axis and insert at each joint; most of these muscles reach the distalmost joint, and only few of them have their terminal insertions in more proximal joints (HESSLER, 1964). No difference between the musculature of late nauplius and the musculature of the adult were noted (HESSLER, 1964). In the second antennal exopod of the cephalocarid *Hutchinsoniella macracantha*, the number of articles increases from 13 to 19 during the postembryonic development (SANDERS, 1963). Comparable increase, although not always identical in number, has been found in other species (see SANDERS & HESSLER, 1964; ADDIS *et al.*, 2007). Despite the lack of actual data, it seems reasonable that new joints are produced together with new intermediate insertions of already existing muscles.

Second antennae. *Branchiopoda.* While the antennal exopod of branchiopod nauplii is generally described as "multisegmented" or "multiannulated", the articulation of this part is not so clear as it may be expected. In *Artemia* the naupliar exopod has two segmentally arranged series of structures: the setae (on the posterior-ventral side) an cuticular folds (on the opposite side). However, their number and position is often not concordant and thus complete joints are lacking (MARUZZO, 2008a) and there is evidence that these two serial elements are rather independent in the whole branchiopod group (MARUZZO, 2008a). Despite the lack of complete joints in the antennal exopod, there are three muscles that run parallel to the proximo-distal axis, inserting at the base of each seta or in the cuticular folds (MARUZZO, 2008a). Concerning number change during postembryonic development among extant branchiopods, only a decrease in the number of cuticular folds (but not of the setae) has been observed (in *Caenestheriella gifuensis* (Ishikawa, 1895); OLESEN & GRYGIER, 2004).

Second antennae. *Ostracoda.* The exopod of podocopan ostracods is usually reduced. Although myodocopid ostracods do not have a naupliar phase (and probably neither an egg-nauplius, cf. WAKAYAMA, 2007), I take here into account their antennal exopod as well. The exopod of myodocopid ostracods is well developed and the musculature was illustrated by KORNICKER (2004) for the adult of *Thaumatocypris rudjakovi* Kornicker, 2004; here some muscles run parallel to the proximo-distal axis, but not detailed observation on their insertions is available. In at least one species, *Thaumatoconcha radiata* Kornicker and Shon, 1976, a one-article increase in the antennal exopod was reported during the postembryonic development (KORNICKER & SHON, 1976).

Second antennae. *Thecostraca.* The naupliar antennal exopod musculature has been described for the first time in this paper. It is very similar to the one described by HESSLER (1964) for cephalocarids (see above).

The number of article in the facetotectan antennal exopod does not change during naupliar development (ITÔ, 1990; KOLBASOV & HØEG, 2003), although the actual number is variable among different species (BELMONTE, 2005).

Ascothoracid naupliar second antennae exopod is usually composed of 6-9 articles, sometimes more (GRYGIER, 1987). While there are few data on ontogenetic sequence, GRYGIER (1993) reported a small ontogenetic increase in article number. In some species the whole second antennae may be reduced or lost (GRYGIER, 1987).

Acrothoracicans have a six-segmented antennal exopod throughout the naupliar phase (TURQUIER, 1967).

In rhizocephalan cirripeds no change in antennal exopod segmentation occurs during the naupliar phase (WALOSSEK *et al*., 1996; RYBACOV *et al*., 2002).

KADO & HIRANO (1994) showed data on article number for the naupliar antennal exopod of 18 species of thoracic cirripeds; in all these cases, an ontogenetic increase was noted. Also MOYSE (1987) noted an increase, one article per moult, for most of the naupliar stages, in *Lepas anatifera*. An increase is also evident from the figures in the papers of COSTLOW & BOOKHOUT (1957 on *Balanus eburneus*, and 1958 on *Balanus amphitrite*), BARNES & COSTLOW (1961 on *Balanus balanus* (Linnaeus, 1758)), DINEEN (1987 on *Lithotrya dorsalis*), YAN

(2003 on *Chinochthamalus scutelliformis*), and YAN & CHAN (2004 on *Chthamalus neglectus*). As far as I am aware, the only exception to this is *Ibla quadrivalvis*, where, according to the figures in ANDERSON (1987), the number of article does not change during the naupliar phase.

Second antennae. *Mystacocarida*. Nothing is known on the muscular system of the appendages of mystacocarids. The second antennae have nine articles from hatching to adult, but the endopod acquired one article (from 5 to 6) during postembryonic development (OLESEN, 2001).

Second antennae. *Copepoda.* The intrinsic musculature of the antennal exopod has muscles spanning more than one article parallel to the proximo-distal axis, but no detailed information on intermediate insertions is available (BOXSHALL, 1985). In the exopod of copepod second antennae, new articles are added only during the naupliar phase or just at the moult from nauplius to copepodid (FERRARI & DAHMS, 2007).

Second antennae. *Remipedia*. Nothing is known on the muscular system of the remiped second antennae; in the naupliar phase the second antennae are biramous and unsegmented (KOENEMANN *et al.*, 2007) and in the adult the exopod is composed of a single large, oval article (e.g., YAGER, 1981).

Second antennae. *Malacostraca.* Few muscles run into the antennal exopod, parallel to the proximo-distal axis in *Sicyonia ingentis*, but no observation on muscle insertions is available (KIERNAN & HERTZLER, 2006). The naupliar second antenna has been described sometimes as unsegmented or with indistinct segmentation throughout the naupliar phase (e.g., RONQUILLO *et al.*, 2006) or unsegmented only in the first naupliar stages, and later with distinct segmentation (HEEGAARD, 1953; FIELDER *et al.*, 1975). In *Penaeus esculentus* Haswell, 1879 the addition of one article in the antennal exopod during the nuapliar phase has been described (FIELDER *et al.*, 1975).

Mandibles. *Cephalocarida*. The musculature of the naupliar mandibular exopod is very similar to that of the second antennae (see above), but it has fewer muscles (HESSLER, 1964). The exopod of the mandible of the cephalocarid *Hutchinsoniella macracantha* has 6 articles in the first naupliar stage; later it atrophies (SANDERS, 1963). Also in other species the exopod atrophies but, while in *Lightiella incisa* Gooding, 1963 it starts from a six-segmented condition as well (SANDERS & HESSLER, 1964), in *Lightiella magdalenina* Carcupino, Floris, Addis, Castelli and Curini-Galletti, 2006 it starts with 7 articles (ADDIS *et al.*, 2007). The exopod may (*L. incisa* and *L. magdalenina*) or may not (*H. macracantha*) pass through conditions with fewer articles than the starting ones.

Mandibles. *Branchiopoda.* Living branchiopods have uniramous mandibles, retaining (not even always) the endopod (OLESEN, 2004).

Mandibles. *Ostracoda.* The exopod is usually very reduced.

Mandibles. *Thecostraca*. The naupliar exopod of the mandibles has been described for the first time in the present paper and, as for the antennal exopod, it is very similar to the one described by HESSLER (1964) for cephalocarids (see above).

As it is for the second antennae, there is no ontogenetic variation in the number of articles in the facetotectan mandible exopod (ITÔ, 1990; KOLBASOV & HØEG, 2003), although there is phylogenetic variation (BELMONTE, 2005).

The mandibular exopod of ascothoracid nauplii is usually composed of 6-9 articles, sometimes more (GRYGIER, 1987). By studying a partial ontogenetic sequence, GRYGIER (1993) did not notice any increase in article number. In some species the whole mandible may be reduced or missing (GRYGIER, 1987).

Acrothoracicans have a six-segmented mandibular exopod throughout the naupliar phase (TURQUIER, 1967).

Rhizocephalan cirripeds do not change their mandibular exopod articulation during the naupliar stages (WALOSSEK *et al.*, 1996; RYBACOV *et al.*, 2002).

Most of what has been said for thoracican naupliar second antennae is valid also for their mandibles. However, the increase in article number is usually much smaller here (usually one article only) since the mandibular exopod have usually less articles then the antennal one. Anyway, only MOYSE (1987; on *Lepas anatifera*) explicitly mentioned this increase in the text, and I have shown in this paper an identical increase in *Balanus improvisus*. Increase is also evident from figures of COSTLOW & BOOKHOUT (1957 on *Balanus eburneus*, and 1958 on *Balanus amphitrite*), BARNES & COSTLOW (1961 on *Balanus balanus*), YAN (2003 on *Chinochthamalus scutelliformis*), and YAN & CHAN (2004 on *Chthamalus neglectus*). Again, as for second antennae, according to the figures of ANDERSON (1987), article number in the exopod of the mandible of *Ibla quadrivalvis* does not increase during ontogeny. This seems also the case for *Megabalanus volcano* and *Megabalanus rosa*, according to the figures of KADO & HIRANO (1994). I have shown that in *B. improvisus* the new joint gets intermediate muscle insertion of already present muscles.

Mandibles. *Copepoda.* The mandibular musculature was described only for adults (BOXSHALL, 1985) and, in the exopod, involved only muscles parallel to the proximo-distal axis. Little is known about copepod mandible development, but the addition of new articles in the madibular exopod has never been observed so far (FERRARI & DAHMS, 2007).

Mandibles. *Malacostraca.* Few muscles run into the antennal exopod, parallel to the proximo-distal axis in *Sicyonia ingentis*, but not observations on muscle insertions are available (KIERNAN & HERTZLER, 2006). The naupliar mandible has always been described as unsegmented throughout the naupliar phase (HEEGAARD, 1953; FIELDER *et al.*, 1975; RONQUILLO *et al.*, 2006).

ARTICLES AND MUSCULATURE IN THE NAUPLIAR APPENDAGES. SUMMARY AND COMPARISONS

Although data are still scattered, some general points can be fixed. It is rare to find muscles confined within an article and usually muscles have intermediate insertions on each joint they pass through. It is also rare to find “transverse” muscles, i.e., muscles that do no run parallel to the proximo-distal axis. First antennae appear to have ontogenetic variation in article number only in ostracods, ascothoracidans, some thoracicans, copepods (post-naupliar phase) and some malacostracans. Second antennal exopod shows ontogenetic variation in article number in cephalocarids, branchiopods (only decrease in the number ofcuticular folds; one species), ostracods (one species), ascothoracidans, thoracicans (with some exceptions), copepods and one malacostracans. Only among thoracicans the number of articles of the mandibular exopod increases (and by one article only).

In many groups, however, there seems to be no ontegenetic variation in article number. Even thus, it is sometimes found that relatively closely related species have different article number. Adult cephalocarid mandibles are palpless and this condition is acquired during postembryonic development. The naupliar mandible is biramous and has an exopod with 6 (*Hutchinsoniella macracantha* and *Lightiella incisa*) or 7 (*Lightiella magdalenina*) articles. In facetotectans no ontogenetic variation has been observed but BELMONTE (2005) noted that article number of second antennal and mandibular exopod is different in different species.

Clearly, these are structures with high diversity in article number, both in ontogeny and in phylogeny. Comparable diversity is not found in the protopod (which usually has one or two articles) or in the endopod (which is usually shorter and sometimes modified) and, mainly, a comparable diversity is only found in some structures (often called “flagellar”) of postmandibular appendages (see below).

It is important to note here that all these structures have a similar muscular arrangement: a few muscles running parallel to the proximo-distal axis through most of the structures (sometimes throughout the whole length of the appendage), with intermediate insertions on each joint. The production of new joints usually involves just the production of new intermediate insertions of already present muscles. A similar condition was described for the antennae of a centipede and for the rami of the pleopods of an amphipod crustacean (MARUZZO, 2008b). It has been described here for the antennal and mandibular exopod of the nauplii of *Balanus improvius* and there is strong evidence that this is also the case for the first antennae of ostracods (but few new muscles may also be produced) and copepods, the second antennae of cephalocarids, the second antennae of myodocopid ostracods, the second antennae and mandibles of many thecostracans.

As noted above the protopod does not show such marked ontogenetic and phylogenetic variation. Its musculature has been described for anostracan branchiopods (BENESCH, 1969; FRYER, 1983; KIERNAN & HERTZLER, 2006) and cephalocarids (HESSLER, 1964) and one or more muscles that are not parallel to the proximo-distal axis of the appendage are always present. Among postmandibular appendages, comparable diversity is found only in the so-called flagellar structures. Flagellar is a function-based definition and is applied to the terminal part of an appendage that has whip-like movements by muscles insert at its base. Flagellar structures can be completely devoid of muscles, as it happens for the antennal flagellum of malacostracan crustaceans and insects (IMMS, 1939), or have some muscles running parallel to the proximo-distal axis, as it happens in the rami of some malacostracan postmandibular appendages (e.g., exopods of pereiopods of lobster larvae, NEIL *et al.*, 1976; rami of the pleopods of amphipods, MARUZZO, 2008b).

ON INTERMEDIATE MUSCLE INSERTIONS AND THE WAY ARTICLES ARE PRODUCED

The articles of the exopod of both second antennae and mandibles are usually regarded as annuli. However, I have shown here that, wherever a detailed study has been performed, muscle insertions have been found on each joint of these structures, and thus, according to the definition of, e.g., BOXSHALL (2004) they are not annuli. A further discussion on the anatomical distinction between true articles and annuli is in MARUZZO (2008b).

All these data support the observations of Maruzzo *et al.* (2007) that true articles with just intermediate muscle insertions can be produced sequentially, as annuli often are. Maruzzo *et al.* (2007), however, did not discuss the evolutionary meaning of this difference between annuli, true articles with intermediate muscle insertions (in the following, TAIs) and all other true articles (in the following, TAs). In this section I discuss how these kinds of articles are produced during ontogeny and what is the evolutionary meaning of these differences.

Basically, there are three developmental periods in which TAs are produced: embryogenesis (or prelarval stage), metamorphosis and anamorphosis. At hatching all the TAs of an appendage are usually present; exceptions are those arthropods with a prelarval stage. The prelarval stage (briefly reviewed in MINELLI *et al.*, 2006) is often described as a “hatched embryo” because it usually moults rapidly into the first larval stage (it lasts only a few hours at most), and because it is not functional. In this stage the appendages often show a reduced number of TAs. Also after a metamorphosis all the TAs of an appendage are usually present. I use here the term “metamorphosis” to refer not only to those of holometabolous insects, but also to any abrupt morphological change an arthropod can go through. In anamorphic development, an arthropod adds now trunk segments and appendages during postembryonic development. The new appendages may have their final number of articles already at their first appearance, or take one-two moults to become complete. In the centipede *Lithobius forficatus* (Linnaeus, 1758) new legs gets their final article number with one or two moults, and before that they are not functional (SCHEFFEL, 1969). Also TAIs and annuli can be produced in the same developmental periods as TAs but, in addition, they can also be produced sequentially during postembryonic development. Examples of sequential postembryonic addition of TAIs have been provided throughout this paper. Examples of sequential postembryonic addition of annuli are, e.g., the antennal flagellum of malcostracans and insects (see MARUZZO *et al.*, 2007).

Different hypotheses can be proposed to explain these difference: there may be developmental constraints on the production of new muscles during postembryonic development. Alternatively, TAs may be produced simultaneously (thus this difference is a simultaneous versus sequential segmentation), or there may be other kinds of developmental or functional constraints.

I have just shown that joints that are produced later in development are either uncoupled from muscles (annuli) or coupled just with new intermediate insertion of an already existing muscle (TAIs). Entirely new muscles connected with joints, thus, tend to develop in a given developmental period (embryogenesis, metamorphosis or anamorphosis). Anyway, most arthropods regenerate appendages very well, as long as they moults, and the regenerated appendage is usually equipped with a normal muscular system (MARUZZO *et al.*, 2005). Also, apparently new muscles are produced during the postembryonic development in the first antennae of ostracod crustaceans (see above).

The production of TAs versus TAIs and annuli is also not strictly correlated to simultaneous versus sequential segmentation. In some cases also TAs may be produced sequentially. Embryological works showed that the formation of, e.g., hemimetabolous insect leg articles is not simultaneous (e.g., NORBECK & DENBURG, 1991). The pereiopods of some pilumnine crabs provide an extreme example. They develop during the first larval (zoeal) phase, initially as buds but later with some segmentation. They are, however, not functional until the next larval phase (megalopa) where they are also complete (CLARK, 2005).

However, he production of TAs restricted to given developmental periods must be due to some kind of constraints. There are, however, two main kinds of constraints: developmental and functional (which is just another way to say that morphological evolution is driven by developmental constraints and natural selection; cf. FUSCO, 2001). Distinguishing between the two is often hard, unless there is the possibility of performing experiments of artificial selection as it has been done, e.g., for evaluating these components in the evolution of allometry between butterfly forewing and hindwing (FRANKINO *et al.*, 2007; see also BRAKEFIELD, 2006 for more arguments on this distinction). Indeed, the developmental periods in which TAs can be produced are actually the period during which the functional morphology of the animal is established or modified. Arthropods can hatch in the prelarval stage, in which some TAs can be missing in the appendages; however, as noted above, the prelarval stage is not functional. "Metamorphosis" has been used to refer to all the abrupt morphological change that different arthropods can go through; it is rather obvious that a change in morphology involves also a change in the way the animal "works". An appendage produced during anamorphic development can take one or two moults to be completed; in the meanwhile, however, it is not functional.

Although the possible existence of some developmental constraints can not be completely ruled out, I think there is enough evidence to conclude that the ontogenetic difference outlined above is due to functional constraints. Indeed, once an appendage is functional, the production of new TA would change its functional mechanism, while the production of new TAIs or annuli would not.

CONCLUSION

The difference outlined by BOXSHALL (2004) in the timing of development as an important divide between production of true articles and annuli is basically a circular statement. Annuli are defined as such because of the way they "work" (see MARUZZO, 2008b) and I have shown here that also the timing of development is due to functional aspects. The observation (MARUZZO *et al.*, 2007) that true articles where intrinsic muscle insertions are intermediate insertions of a muscle can develop more like annuli than true articles simply reflects the fact that TAIs and annuli have very similar function, and neither of the two can move the appendage independently from other articles as TAs can do.

So, there is currently no evidence to support any foundamental developmental difference between true article and annuli. Different developmental processes can obviously be responsble for the observed differences between true articles and annuli of a given appendage in a given taxon, as it may be the case for insect tarsomeres versus other leg articles (see introduction). However, any generalized developmental differences between TAs and TAIs and annuli of different appendages and different taxa relies, at the moment, on aspects of functional morphology, the same aspects that defined these articles.

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ON THE ANATOMY OF SOME ARTICLES IN ARTHROPOD APPENDAGES

ABSTRACT

The musculature of the rami of the pleopods of the amphipod *Gammarus roeselii* and of the antennae of the centipede *Lithobius forficatus* is described. In both of these appendages there are few muscles that run parallel to the proximo-distal axis of the appendage, with intermediate muscle insertions at each joint. Both of these appendages add new articles during postembryonic development and these are coupled with new intermediate insertions of already present muscles. Traditionally, the articles of the pleopodal rami of amphipods are considered annuli, while the articles of the centipede antennae, true articles. This difference is explained here as a difference in the structure of the joints and of the muscle insertions. These differences are important in terms of functional morphology, but are misleading for evolutionary ones; annuli and true articles are often conceived as different structures but it is shown here that there is not clear-cut qualitative difference between the two. The occurrence of "terminal" and "intercalary" annulations as well as the possible evolutionary origin of these differences is discussed.

INTRODUCTION

While not all arthropods have well-jointed (i.e., divided in articles) appendages, the presence of well-jointed appendages is usually considered a primitive condition of arthropods. Indeed, arthropod literally means "jointed feet". A widely used distinction among the kinds of articles in an arthropod appendage is between "true articles" and "annuli". This distinction is so used in comparative morphology that is even mentioned in invertebrate zoology textbooks (e.g., BRUSCA & BRUSCA, 2003). The difference between true articles and annuli is often claimed to be based on the anatomy of the muscular system, however there is no one clear, widely accepted definition. Snodgrass defined true articles as "any part of an appendage independently movable in some member of the Arthropoda by muscles inserted on its base" (SNODGRASS, 1935: 85). Students of arachnids (e.g., SHULTZ, 1989; VAN DER HAMMEN, 1989) use a slightly different terminology and, following COUZIJN (1976), refer to eudesmatic and adesmatic joints, with the former defined as joints where at least one muscle inserts, and the latter as joint with no muscle insertion. One recent definition states that "true segments [articles] are characterised by the presence of intrinsic muscles that originate, insert, or attach within each segment [article]. By contrast, annuli lack intrinsic muscle origins, intermediate attachments or insertions, although intrinsic muscles or their tendinous extensions may pass through annuli to a more distal insertion site" (BOXSHALL, 2004: 255). Boxshall also proposed a distinction between terminal and intercalary annulations [annuli], considering terminal annulations the articles devoid of any muscle and intercalary annulations those with muscle(s) or tendon(s) passing through. Terminal annulations are always restricted to the distalmost part of appendages and the whole structure with terminal annulations is called terminal flagellum (BOXSHALL, 2004). The phylogenetic distribution of annuli in different appendages has been also reviewed by BOXSHALL (2004).

MARUZZO (2008a) noted that the exopod of both second antennae and mandibles of many different crustacean nauplii, which are usually described as "annulated", are not composed of annuli. Indeed, each joint of these structures has muscle insertions, which usually are intermediate insertions of muscles that, running parallel to the proximo-distal axis of the appendage, originate in more proximal articles and have their terminal insertions in more distal ones. To ascertain the actual anatomical difference between true articles and annuli, I study here two models, one generally described as composed of annuli and one of true articles, for both of which there was good evidence for the presence of a few muscles running throughout the appendage, parallel to the proximo-distal axis.

The two models are the pleopods of the malacostracan *Gammarus roeselii* Gervais, 1835 (Crustacea, Amphipoda) and the antennae of the myriapod *Lithobius forficatus* (Linnaeus, 1758) (Chilopoda, Lithobiomorpha). As a rule for gammaridean amphipods, the first three pairs of appendages (pleopods) of the abdomen (pleon) of *G. roeselii* are biramous, with a proximal peduncle and two rami (exopod and endopod) generally described as annulated. Observations on the musculature of this species are lacking but in other amphipods it is known there are few muscles (two in, e.g., *Eurythenes gryllus* (Lichtenstein, 1887); BOUDRIAS, 2002) running throughout each ramus; however, there are not specific studies on the insertion pattern of these muscles. The antennae of *L. forficatus* are composed of articles generally described as true articles. There are four muscles in each antennal article (named flexor, extensor, dorsal retractor and ventral retractor by IMMS, 1939; very similar observations were also reported for another centipede, see APPELGARTH, 1952); a slightly different condition is limited to the two most proximal articles, where one muscle (possibly corresponding to the dorsal retractor of the following articles) does not run parallel to the proximo-distal axis. IMMS (1939) was not clear about the individuality of these muscles, he did not state explicitly if, with the possible exception of the two most proximal articles, there are just four muscles going throughout the antenna and inserting on each joint (as it seems from his figure) or each article has four muscles which are not continuous with the corresponding ones in the proximal and distal articles. Both models studied here also add new articles during the whole postembryonic development. The mechanism of new article production has been described for the first postembryonic stages in the antennae of *L. forficatus* by SCHEFFEL (1969) and for the whole life in the pleopods of *G. roeselii* by MARUZZO (2008b).

MATERIALS AND METHODS

Specimens of *G. roeselii* were collected in Vicenza (Italy; material from the same population was also used in a different work, MARUZZO, 2008b); eventually, females with the brood pouch were raised in laboratory (conditions similar to those described for the isopod *Asellus aquaticus* (Linnaeus, 1758) in MARUZZO *et al.*, 2007) and newly released specimens were studied. Specimens of *L. forficatus* from laboratory rearing were kindly provided by Magda Biasiolo and Francesca Bortolin. Specimens were fixed in 10% formalin, dissected parts of interest were embedded in Paraplast Plus, sectioned at 7-10 μm and stained with haematoxylin-eosin. The bigger and more sclerotized specimens of *L. forficatus* were treated with chitinase (Sigma; 0.7 u/ml in 0.2M hepes buffer (pH 7.4) at 37°; overnight to three days, time depending on the size) to soften the cuticle before embedding.

RESULTS

THE PLEOPODS OF *GAMMARUS ROESELII*

Two muscles run throughout each ramus of the pelopods of *G. roeselii*; in addition, in the exopod there is a small, fan-shaped muscle, which runs obliquely in the proximal part of the first article (Fig. 6.1). Both muscles running throughout each ramus are localized in the posterior side of the ramus and have broad, intermediate insertions, rich in tendon matrix, at each joint (Fig. 6.2) and a terminal insertion at the last joint. Arthrodial membrane is present at each joint on the side where muscles are attached but it is as much as lacking on the opposite side, although the sclerites of the different articles are easily identified (Fig. 6.2A). Production of new joints is always coupled with the formation of new intermediate insertions of these two muscles.

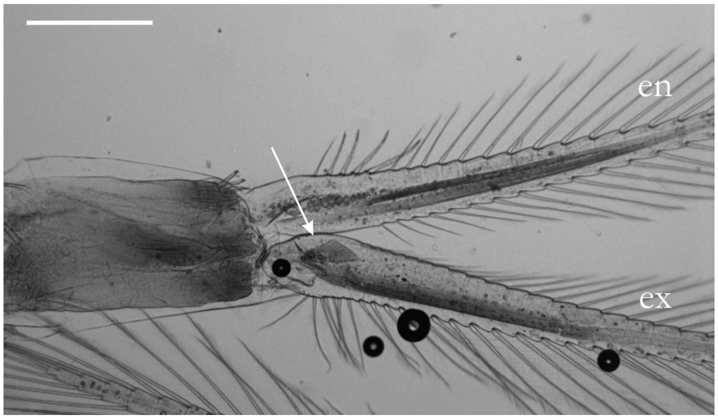

Fig. 6.1. Pleopod of *Gammarus roeselii*. The exopod (ex) and endopod (en) with their two muscles are shown; the small muscle in the first article of the exopod (arrow) is also shown. Scale bar 500 μm.

THE ANTENNAE OF *LITHOBIUS FORFICATUS*

The intrinsic musculature of *Lithobius forficatus* antennae was already described by IMMS (1939) and the present account basically confirms his observations. As noted in the introduction, IMMS (1939) was not clear about the individuality of the muscles, i.e., if there are just four muscles running throughout the antenna and inserting at each joint, or each article has four separate muscles. With the exception of the first two articles, there are four muscles running throughout the antenna and inserting at each joint (Fig. 6.2C-F). Young specimens have muscles composed of few bundles (Fig. 6.2C). Adults have much bigger muscles, each composed of several bundles (Fig. 6.2D-F). Extensive arthrodial membrane is present on all sides of all joints, always more extensive than the membrane present on the side of the muscles in the pleopodal rami of *G. roeselii*, and it also allows telescopic movements, as it is clear comparing Fig. 6.2C with 6.2D-F. The production of new joint is accompanied by with new intermediate insertions of the pre-existing muscles.

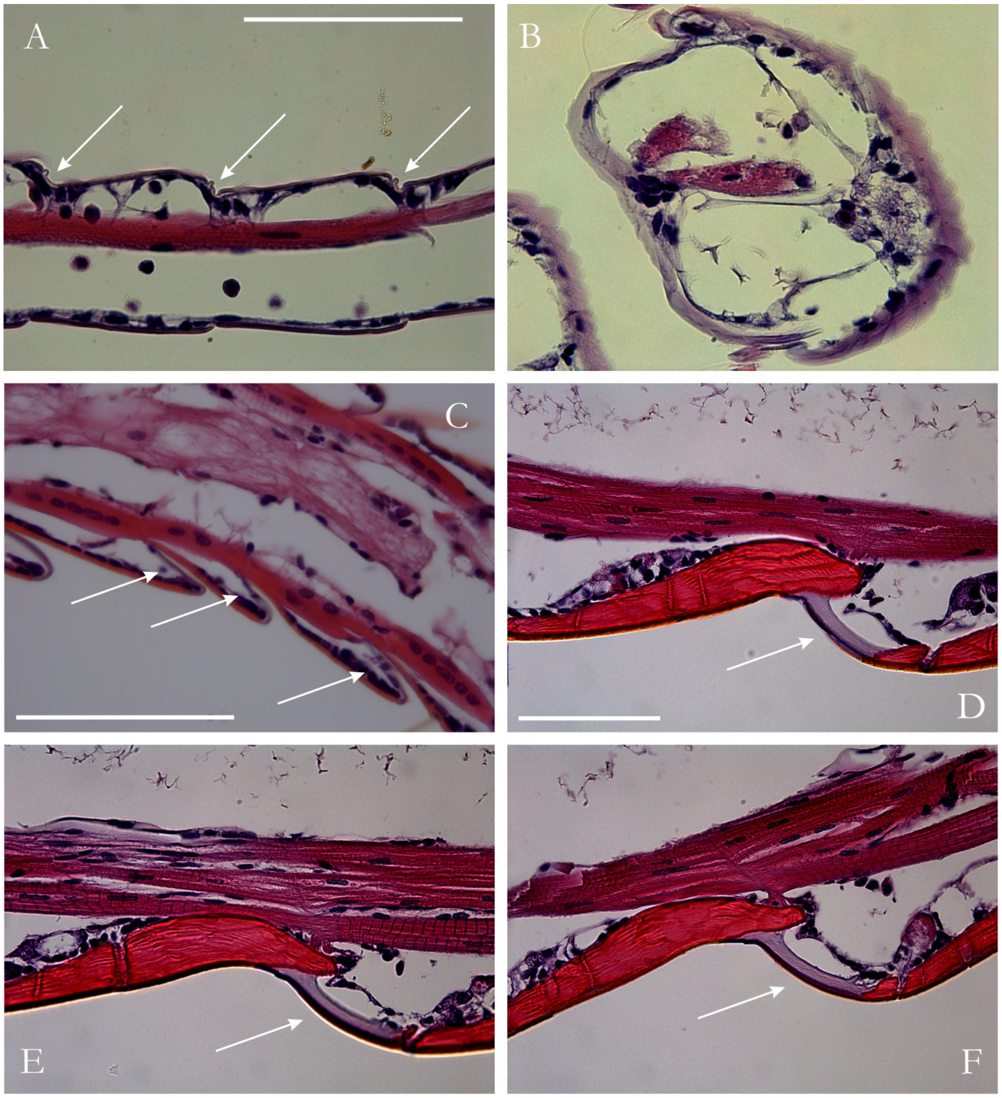

Fig. 6.2. The musculature of the rami of *Gammarus roeselii* pleopods and of the antennae of *Lithobius forficatus*. A and B, section of the rami of *G. roeselii* pleopods. A, longitudinal section showing the intermediate insertions of a muscle in the joints. B, cross-section showing the two muscles located in the posterior side. C, D, E, and F, longitudinal sections of the antennae of *Lithobius forficatus*. C, a juvenile of second stadium. D, E, F, same muscle and same joint of an adult; E is a section 40 µm after D and F after 80 µm. Arthrodial membranes of the joints are indicated by arrows. Scale bars (B = A; E and F = D) 100 µm.

DISCUSSION

TRUE ARTICLES AND ANNULI

Between the rami of amphipod pleopods and the centipede antennae described here there are both similarities and differences. Both appendages are equipped with few muscles that run throughout a part of the appendage with intermediate insertions at each joint they pass through, but the structure of both intermediate muscle insertions and joints are different.

The general morphology of an arthropod muscle insertion is far from uniform, and this is true also for different muscle insertions in the same animal. This diversity lies not only in the number of the muscles that can insert in a point, but also in the presence and extent of an apodeme (integumentary ingrowth) and of a tendon matrix (BITSCH & BITSCH, 2002). The intermediate muscle insertions described here for the pleopods of *G. roeselii* are broad and rich in tendon matrix; by contrast, the intermediate muscle insertions described for the antennae of *L. forficatus* are narrow, strictly localized in the proximal part of the sclerites of each article, and with basically no tendon matrix.

The extent of arthrodial membrane is also very different. The arthrodial membrane can be defined as the unsclerotized part of the cuticle between two trunk or appendage sclerites. Clearly, the relative movements between two contiguous trunk segments or appendage articles are dependent on the amount of arthrodial membrane present. In the rami of the pleopods of *G. roeselii* there is little arthrodial membrane where the muscles insert; on the opposite side, an arthrodial membrane is almost absent; by contrast, there is extensive arthrodial membrane all around each joint in the antennae of *L. forficatus*, and this arthrodial membrane can even allow remarkable telescopic movements.

These differences between the two models are important for the relative movements each article can perform, but only rely on quantitative aspects, i.e., abundance of tendon matrix and extension of arthrodial membrane. As noted in the introduction, the articles in the rami of the pleopods of *G. roeselii* are usually considered annuli, while those in the antennae of *L. forficatus* are classified as true articles. This difference, however, does not reflect a qualitative difference as it is often assumed, but only reflects the different functional morphology of these articles. BOXSHALL (2004; followed later by MARUZZO *et al.*, 2007) outlined also a difference in the timing of development between true articles and annuli; however, MARUZZO (2008a) showed that even this difference is not due to a basic developmental difference between these articles, but it is due to the same aspects of functional morphology that produced the distinction between true articles and annuli.

Annuli and true articles are often conceived as different structures in comparative morphological studies. But, while from a functional morphology point of view this is surely correct, for an evolutionary discussion this is inappropriate. There is no evidence for any clear-cut anatomical or developmental difference between true articles and annuli. Thus any discussion that involves the distinction between true articles and annuli of different appendages and/or different arthropod groups should take this point into account.

TERMINAL AND INTERCALARY ANNULATIONS

The distinction between terminal and intercalary annulations (see introduction) needs further discussion. Clearly, terminal annulations are anatomically well recognizable since are the terminal part of an appendage with well-formed articles but no muscles. The phylogenetic distribution of terminal annulations, however, is very restricted. They are only found in the antennal flagellum of the first and second antennae of malacostracan crustaceans and insect (IMMS, 1939), in the first antennae of some branchiopods (e.g., in *Caenestheriella australis* (Loven, 1847) if the lobes present on one side of the first antennae are interpreted as modified articles; BOXSHALL, 2004) and in the ventral ramus of remipede first antennae (BOXSHALL, 2004); this may even be due to a common evolutionary origin since, according to some recent phylogenetic hypotheses, these groups (or at least some of them) are closely related (e.g., REGIER *et al.*, 2005; HARZSCH, 2006; MALLATT & GIRIBET, 2006). Terminal annulations have also been reported for the exopod of the pereiopods of anaspidacean crustaceans (BOXSHALL, 2004), but this is likely an apomorphic character for this group, since exopods of other malacostracans thoracic appendages have muscles that run, parallel to the proximo-distal axis, throughout the exopod (SCHMIDT, 1915; NEIL *et al.*, 1976).

The existence of intercalary annulations as defined by, e.g., BOXSHALL (2004) must now be discussed. I have shown (present paper for the pleopodal rami; MARUZZO, 2008a for the exopod of the naupliar appendages) that some structures usually defined as "annulated" have indeed joints with intermediate muscle insertions. "Intercalary annulations" have been described in several other arthropod appendages; for example, in many caridean pereiopods the carpus is often "annulated". However, in the only work of which I am aware where the musculature has been studied, each article of the carpus was shown to have three intrinsic muscles (READ *et al.*, 1991), thus neither these articles should be defined annuli.

As noted above "annulus" is a definition pertaining functional morphology, so it should not be surprising that, while a clear definition of annuli has been stated to involve the presence/absence of intrinsic musculature, most of the times annuli have been named as such just by observation of their movements, without any consideration of the muscular system. While I suspect that a close study on the muscular system would discover unexpected muscle insertions in many structures traditionally defined as having intercalary annulations, in some of them the absence of any muscle insertion seems well established. I am referring here to, e.g., the tarsus of insect legs and the first walking leg of whip spiders. I want here to note that these structures 1) are in the terminal portion of an appendage and 2) have been described as having a tendon (not a muscle) passing through (e.g., SNODGRASS, 1935; SHULTZ, 1989). Thus, all these observations, although still fragmentary, suggest that joints without muscle insertions are only those that have either no muscle passing through or just tendon(s). If this will turn out to be true, a possible developmental coupling between the formation of joints and the formation of muscle insertion (if muscles are present) will have to be investigated.

ONE MUSCLE FOR EACH OR ONE MUSCLE FOR ALL?

Describing the muscular system of the antennae of *L. forficatus*, IMMS (1939) did not explicitly describe four muscles going throughout the antenna with intermediate insertion on each joints (as I have shown here it is the case), but described every article to have four segmentally arranged muscles. This difference may seem trivial but it recalls the way by which muscles are produced within an appendage. In the pereiopods of early lobster embryos, two muscle precursors (thin and long multinucleate cells) are present throughout the endopod. Later in development, these muscle precursors subdivide into several units confined to each article and these two primordial muscles produce the pairs of antagonist muscle of each article (S. HARZSCH & S. KREISSL, unpublished data). Although this mode of muscle development may be peculiar to lobster appendages, it is tempting to propose that the difference between a group of articles with few muscles running through them with intermediate insertions at each joint and a group of articles each equipped with "its own" antagonistic muscles may be the product of heterochronic change.

ACKNOWLEDGEMENTS

Magda Biasiolo, Francesca Bortolin and Leandro Drago helped in different ways to perform this study. Steffen Harzsch and Sabine Kreissl kindly shared with me their unpublished data on muscle development in lobster appendages. Alessandro Minelli and Giuseppe Fusco provided useful comments on an earlier draft of this paper.

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ON THE PROXIMAL “GROWTH ZONE” OF ARTHROPOD APPENDAGES

ABSTRACT

Detailed observations are already available for the production of new articles during postembryonic development in the second antennal flagellum of the isopod *Asellus aquaticus*. To compare this model with another appendage that increase in article number during postembryonic development, I here described how new article are produced in the rami of the pleopods of the amphipod *Gammarus roeselii*. The development of the epidermis in both *A. aquaticus* antennae and *G. roeselii* pleopods was also investigated using nuclear staining and confocal microscopy. New articles on the amphipod pleopodal rami are produce by the division of the more proximal one (called meristematic article) only. In the epidermis, mitoses are found only in proximal part of the rami, although not just within the meristematic article. Cells produced in the proximal part are continuously moved distally by the production of new cells and, at the same time, they differentiate into general epidermal cells becoming longer and thinner. The development of the epidermis in the second antennal flagellum of the *A. aquaticus* is very similar, only the cell become longer and thinner than that of the pleopodal rami of *G. roeselii*. The occurrence and evolutionary origin of comparably similar “growth zone” in arthropod appendage is discussed.

INTRODUCTION

In vertebrates the cellular events involved in appendage development are relatively well understood (e.g., FERNÁNDEZ-TERÁN *et al.*, 2006 and references therein) and this knowledge is important in order to understand both the actual function of some developmental genes and the evolution of the development process of vertebrate appendages, i.e., to understand the basis of vertebrate appendage morphological diversity (e.g., HINCHLIFFE, 2002; NEWMAN & MÜLLER, 2005).

Arthropod appendages exhibit a huge morphological diversity (e.g., HANSEN, 1925, 1930; BOXSHALL, 2004). Although “research into the evolution of arthropod appendage development is still very much at its beginning” (PRPIC & DAMEN, 2008: 393), there are several interesting insights coming mainly from comparative developmental genetics (see review and discussion in, e.g., WILLIAMS & NAGY, 1996, 2001; NAGY & WILLIAMS, 2001; HEMING, 2003; KOJIMA, 2004; WILLIAMS, 2004; ANGELINI & KAUFMAN, 2005; GIORGIANNI & PATEL, 2005; PRPIC & DAMEN, 2008). Specific studies on the cellular events involved in arthropod appendage development are, however, very scarce. Indeed, even for *Drosophila* appendages some important aspects of development at cellular level have been elucidated only in the last few years (see HE & ADLER, 2001 for the antennae; MIRTH & AKAM, 2002 and TAYLOR & ADLER, 2008 for the legs; BAENA-LÓPEZ *et al.*, 2005 and TAYLOR & ADLER, 2008 for the wings). Concerning non-insect arthropods the most recent studies on cellular aspects of appendage development deal with branchiopod phyllopods (FREEMAN *et al.*, 1992; WILLIAMS & MÜLLER, 1996; FREEMAN, 2005; WILLIAMS, 2007). The paucity of this knowledge severely limits our understanding of morphological evolution of arthropod appendages (WILLIAMS, 2004).

Malacostracan crustaceans and insects have antennae of similar structure: a proximal peduncle with articles provided of intrinsic muscles is followed by a distal flagellum devoid of muscles (IMMS, 1939). For the malacostracans this is true for both the first and the second antennae and also for antennae with more than one flagellum. Recently, MARUZZO *et al.* (2007) have described in detail the mechanism of postembryonic article production in the flagellum of the second antennae of a malacostracan species, the isopod *Asellus aquaticus* (Linnaeus, 1758). The flagellum of the second antennae of *A. aquaticus* is mostly composed of “quartets”, series of four articles with a distinctive setal pattern; the part of the flagellum made by quartets was called “central region”. The flagellum has an indeterminate growth and produces new articles (and new quartets) during all postembryonic development. All new articles and quartets are produced in the proximal part of the flagellum (the “meristematic region”): the first article (the meristematic article) keeps on giving off new articles; these (“one-article incomplete quartets”) in turn divide - rather independently from each other - three more times, producing four final articles (a “complete quartet”). The distalmost articles (collectively, the “apical complex”) are not arranged in quartets. An undamaged flagellum has always a five-article apical complex (MARUZZO *et* a*l.*, 2007), following regeneration from the peduncle there is always a four-article apical complex (MARUZZO *et al.*, 2007) and following amputations along the flagellum many different kind of apical complexes can be produced (MARUZZO *et al.*, in press). This mechanism of production of new articles is similar in the antennal flagellum of other isopods as well as in other malacostracans (first antennae included) and insects, the main evolutionary variable apparently being the number of division the articles produced by the meristematic one will later go through (MARUZZO *et al.*, 2007; MARUZZO, 2008a); so, new articles are always produced in the proximal part of the flagellum and the first flagellar article is always the site where segmentation starts.

At the cellular level, HAAS (1955) investigated to some extent the production of new articles in the antennae of the cockroach *Periplaneta americana* (Linnaeus, 1758), and found that mitoses are localized in the proximal part of the flagellum, where new articles are also produced. Although focusing on the development of the peripheral nervous system, a “proximal proliferation zone” was also detected in the lateral flagellum of the first antennae of the spiny lobster (HARRISON *et al.*, 2001; DERBY *et al.*, 2003).

Not only the antennal flagellum, however, increases in article number during postembryonic development. Different appendages of copepod crustaceans increase their article number during postembryonic development and, in these appendages, a “source segment”, defined as “a segment from which a limb is patterned by the formation of new segment elements”, has often been observed (FERRARI & DAHMS, 2007). Also other arthropod appendages add new articles during postembryonic development. An example is the pleopods of gammaridean amphipod crustaceans. Gammaridean amphipod crustaceans have three pairs of pleopods (also called “swimmerets”) on the three anteriormost abdominal trunk segments (pleonites; the three more posterior pleonites bears appendages that are usually called uropods; CALMAN, 1909; SCHRAM, 1986; MCLAUGHLIN, 1980). The pleopods are biramous appendages composed of a peduncle that bears two flagellar rami called exopod (the outer one) and endopod (the inner one); both rami have two muscles running throughout their length (BOUDRIAS, 2002; MARUZZO, 2008b). Morphology and development of pleopodal rami, which are usually described as multiannulated, have been little studied. Although never specifically stated, the number of articles in the pleopodal rami increases during postembryonic development: pleopods of adult specimens are usually depicted with many articles (e.g., SCHRAM, 1986; BOUDRIAS, 2002), while newly born specimens have just few. For example, in *Orchestia cavimana* Heller, 1865, the pleopodal rami have one (the endopod) and two (the exopod) articles at hatchling (UNGERER & WOLF, 2005). Anyway, so far the segmentation of the pleopods has been ignored in studies on postembryonic development (see, e.g., SEXTON, 1924; GEISLER, 1994).

To understand if a “growth zone” somehow comparable to that of the antennal flagellum exists also in other appendages, I investigated article production and the development of the epidermis during postembryonic development of the pleopods of a gammarid amphipod (*Gammarus roeselii* Gervais, 1835). In addition, since the mechanism of article production during postembryonic development in the flagellum of the second antennae of *Asellus aquaticus* is well understood, but nothing is actually known about the corresponding development of the epidermis, this aspect is also studied here.

MATERIALS AND METHODS

Specimens of *G. roeselii* and *A. aquaticus*, collected in Vicenza (Italy; the same site where specimens of *A. aquaticus* were collected for previous studies; MARUZZO *et al.*, 2007, in press), were kept in laboratory and fixed at need. Females with brood pouch were isolated and juveniles were subsequently fixed at need.

The external morphology of *G. roeselii*’s pleopods was studied with light and scanning electron microscopy (SEM). Pleopods of specimens fixed in either 4% paraformaldehyde in PBS or 70% ethanol were mounted in ethylene glycol and observed with light microscopy or dehydrated in a graded ethanol series, dried with hexamethyldisilazane (Sigma) (NATION, 1983), coated with gold and observed with a Cambridge Stereoscan 260 scanning electron microscope.

The development of the epidermis was studied in specimens fixed in a 3:1 mix of ethanol and acetic acid (a modified version of the Carnoy’s fixative), and transferred to 70% ethanol where the appendages of interest were usually dissected. Following rehydratation and washing in PBS, samples were incubated for at least 1 hour in PBS plus 0.3% of Triton X-100, treated with Rnase (500 µg/ml in PBS for 30 minutes) and nuclei were then stained with propidium iodide (2.5 µg/ml in PBS for 1 hour). Following washing in PBS, samples were mounted in gel mount aqueous mounting medium (Sigma) or in buffered glycerol anti-fading (90% glycerol, 0.5% n-propyl-gallate in PBS). Observations were made with an epifluoresence microscope and with a confocal laser scanning microscope (CLSM).

RESULTS

PRODUCTION OF NEW ARTICLES IN THE RAMI OF THE PLEOPODS OF *G. ROESELII*

A general view of the pleopods in the amphipod body is shown in Fig. 7.1. Newly born specimens have two and three articles in the pleopodal endopod and exopod, respectively. Subsequently, there is an indeterminate addition of articles, the actual number of articles is usually hard to determinate because of “sign of segmentation”, “incipient” joints and just produced (but not yet well defined) joints in the proximal part of the ramus (see later). Since some arbitrary judgments were impossible to avoid, the actual article number provided below must be taken cautiously. The maximum article number recorded was 23, the exopod has always more articles than the endopod, it usually has 3-4 articles more but a maximum of 6 have been observed. Left-right asymmetry is not rare, and often one ramus (left or right; there is no directional asymmetry) has 1-2 articles less than its controlateral. While no differences are usually found between the pleopodal rami of the first and second pleonites (although sometimes a difference of 1-2 articles can be found between corresponding pleopodal rami), the rami of the third pleopodes have usually 1-2 articles less.

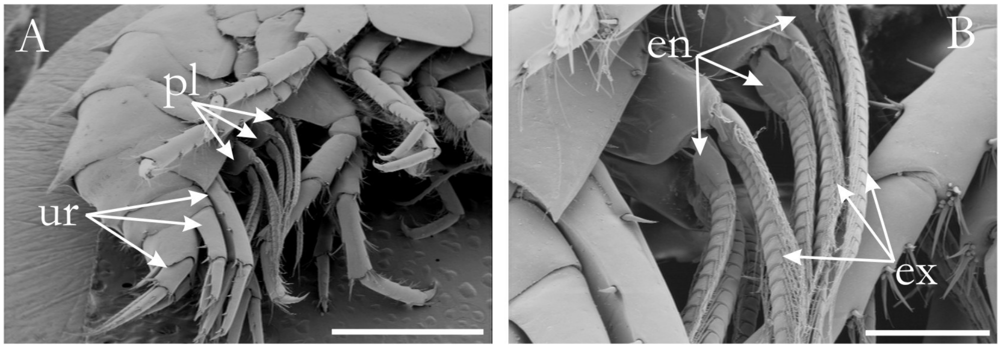

Fig. 7.1. General view of the pleon and pleopods of *Gammarus roeselii*. SEM. A, general view showing pleopods (pl) and uropods (ur); scale bar 2 mm. B, higher magnification of the pleopods in A showing the exopods (ex) and endopods (en) of the three right pleopods; scale bar 500 µm.

Each article of the rami, except the most proximal (in the following called meristematic article) and the terminal ones, has two plumose setae in lateral position (Fig. 7.2A). The terminal article is small and has a different morphology, with two setae in a terminal position (Fig. 7.2A). The meristematic article can have a different number of setae; in the endopod only this article has also some bifid setae (Fig. 7.2B) whose number increases ontogenetically.

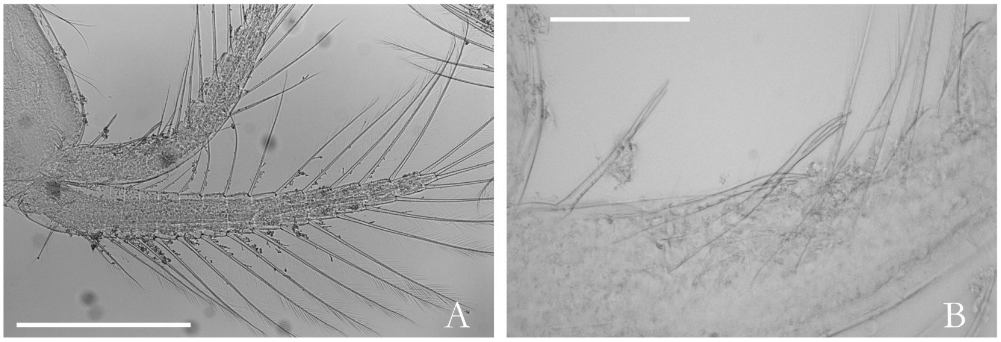

Fig. 7.2. The rami of pleopod of *Gammarus roeselii*. A, complete view of the exopod and proximal part of the endopod, scale bar 500 µm. B, higher magnification on the base of the endopod, showing the bifid setae, scale bar 100 µm.

New articles are produced at the base of the rami, only by the division of the meristematic one. Evidence for this mechanism are the following. a) articles, except the meristematic and the terminal, are in a proximo-distal gradient of length (Fig. 7.2A), as it is expected (and as it happens in *Asellus* and cockroach antennae) if articles are produced small and then lengthen. b) two lateral plumose setae are always present on each article (meristematic and last one being an exception; Fig. 7.2A); I never found any article without setae (as it happens in *Asellus* when an article other than the meristematic one divides) and this is because a varying number of these lateral setae are already produced within the meristematic article and these setae will be later “delivered” to the newly formed articles. On the inner side of the endopod, as mentioned before, there are some bifid setae, whose number increases during postembryonic development; distal to these setae there are the varying number of future lateral plumose setae of the articles. c) at the base, and only there, “indistinct” or “incomplete” joints are usually found (Fig. 7.3).

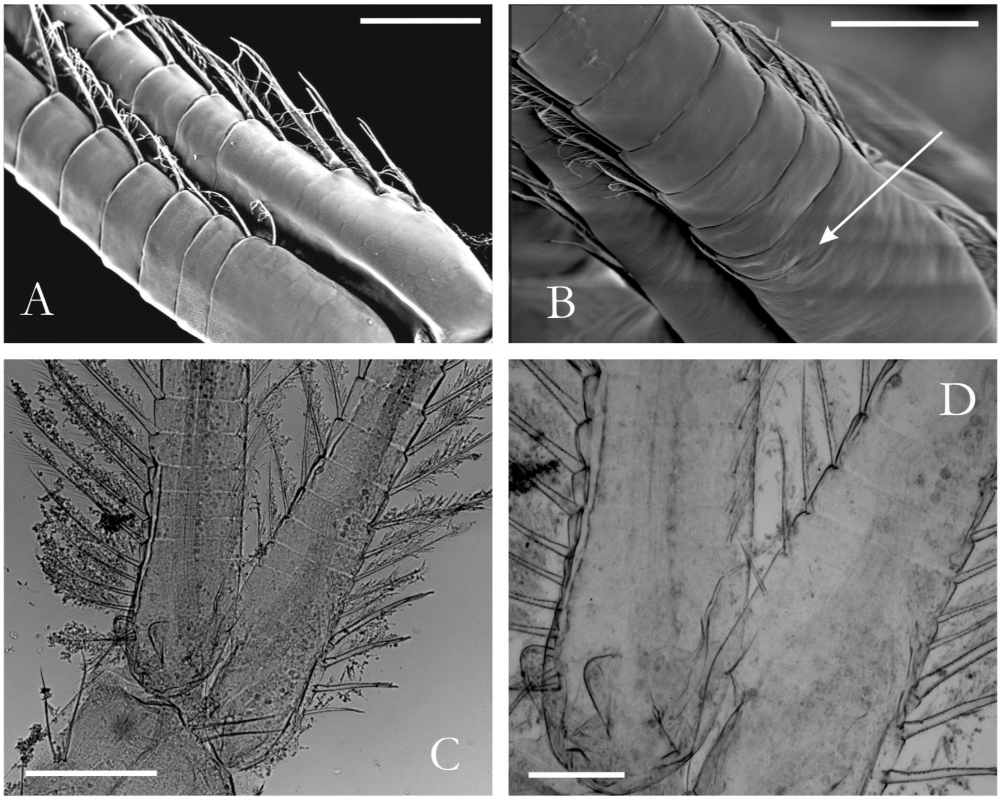

Fig. 7.3. Proximal part of the rami of the pleopods of *Gammarus roeselii*. A, lateral view of the two rami; SEM; scale bar 100 μm. B, an endopod; note an “incomplete” article at the base (arrow); scale bar 100 μm. C, the two rami observed with light microscopy; scale bar 250 μm. D, higher magnification of C; scale bar 100 μm.

DEVELOPMENT OF THE EPIDERMIS IN THE RAMI OF THE PLEOPODS OF *G. ROESELII*

No difference in the postembryonic development, except for those noted above on article number, were observed among the three pairs of pleopods or between the two rami of the same pleopod. An overview of the two rami and a higher magnification view of the proximal, central and distal part of the exopod of a specimens stained with propidium iodide and observed with CLSM is provided in Fig. 7.4. The development of the epidermis is rather clear. In the proximal part the

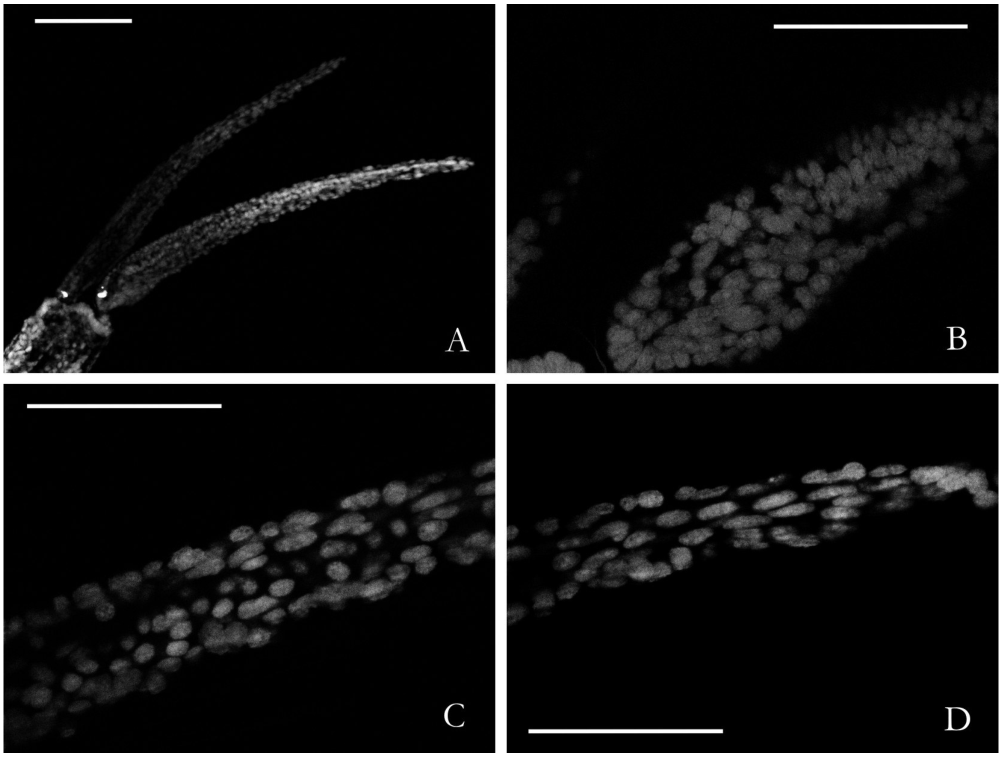

Fig. 7.4. Nuclear staining of the pleopods of *Gammaruc roeselii* observed with CLSM. A, the whole pleopod, endopod is above and exopod below; the picture is a maximum intensity projection; scale bar 100 µm. Detail of the proximal (B), central (C) and distal part (D) of the exopod is shown. The pictures in B, C and D are a single slide of a stack; scale bar 50 µm.

nuclei of the epidermal cells are very close to each other (Figs. 7.4B, 7.5), often irregularly compressed (Figs. 7.4B, 7.5) and mitoses are abundant (a clear example in Fig. 7.6). Moving toward more distal regions, nuclei become more and longer (Fig. 7.4C, D). While not restricted within the meristematic article, mitoses were never detected more than one-two articles distal to it.

Summing up, the epidermis of the pleopodal rami of *G. roeselii* has a proximal proliferation zone, cells are then moved distally by the production of new cells proximally and, going to more distal position, they undergoes a shape change.

DEVELOPMENT OF THE EPIDERMIS IN THE SECOND ANTENNAL FLAGELLUM OF *A. AQUATICUS*

The development of the epidermis of the antennal flagellum of *A. aquaticus* is very similar to that just described for the pleopodal rami of *G. roeselii*. In the proximal part the epidermal cells are very close to each other with more or less rounded nuclei and abundant mitoses (Fig. 7.7). Moving to more distal regions, cells become more and more spaced and the nuclei become longer; the only major difference with *G.roeselii* is that toward the distalmost part the nuclei become much thinner and longer (Fig. 7.8). Mitoses are not restricted within the meristematic article but, anyway, they were not detected within the whole meristematic region, but just in few articles close to the meristematic one.

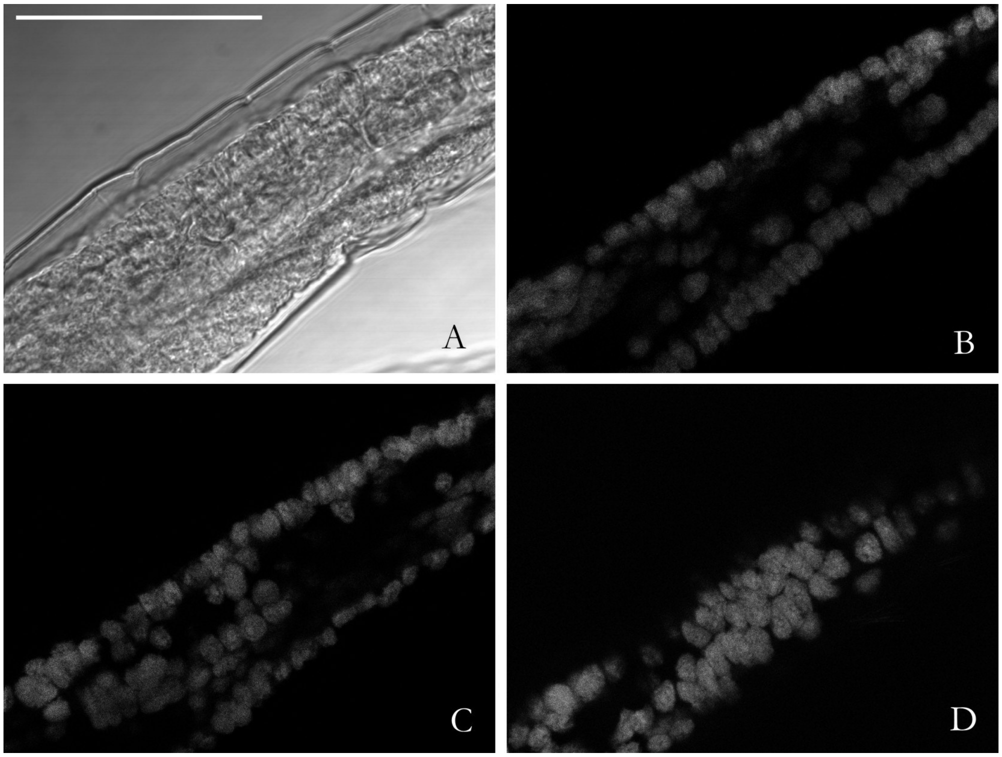

Fig. 7.5. Proximal part of a pleopod exopod of *Gammarus roeselii*, external morphology and nuclear staining. A bright field view (A) and three single slides of a CLSM stack (B, C and D) at different focus levels are shown. Scale bar 50 µm.

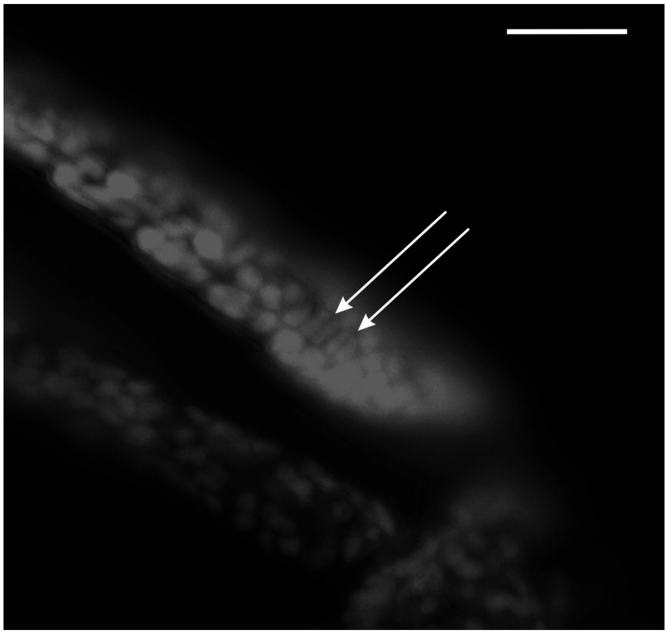

Fig. 7.6. Proximal part of the two rami of a *Gammarus roeselii*, nuclear staining observed with an epifluorescence microscope. Arrows indicate mitotic figures. Scale bar 50 µm.

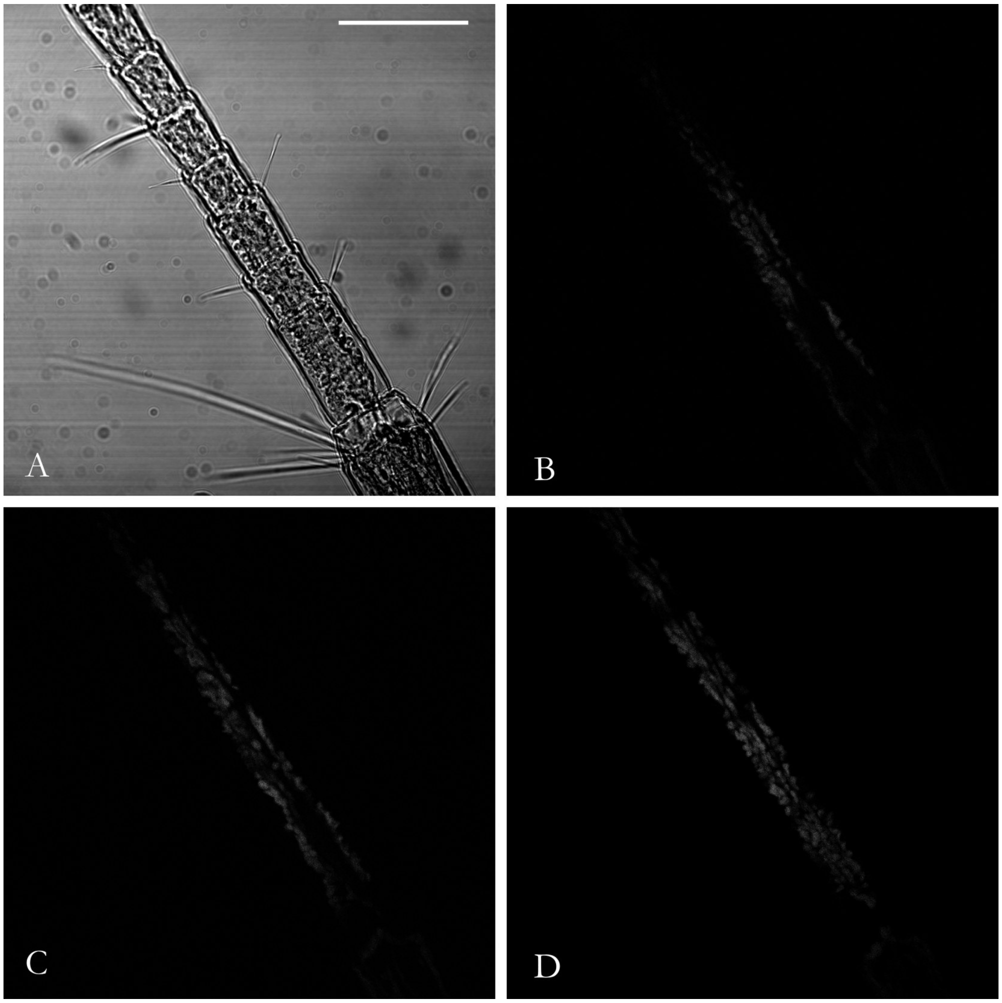

Fig. 7.7. Proximal part of the antennal flagellum of *Asellus aquaticus*, external morphology and nuclear staining. A bright field view (A) and three single slides of a CLSM stack (B, C and D) at different focus levels are shown. Scale bar 50 µm.

DISCUSSION

DEFINING THE GROWTH ZONE

During the postembryonic development of the structures described here new articles are produced and mitoses of the epidermal cells are localized in the proximal part; here, thus, two aspect which in the arthropod trunk are not always clearly connected, growth and segmentation, appears intimately related. Similarities in the mechanism of article production will be discussed later; concerning the developments of the epidermis, there have been just few studies so far. A proximal proliferation zone (a zone where mitoses are localized), however, has been detected also in the cockroach antennae (HAAS, 1955) and in the lateral flagellum of the first antennae of the spiny lobster (HARRISON *et al.*, 2001; DERBY *et al.*, 2003).

The presence of this proximal proliferation zone is connected not only with the production of new articles, but also with the lengthening of the appendage. In both the antennal flagellum of *A. aquaticus* (MARUZZO D., unpublished data) and the antennae of cockroach (CAMPBELL & PRIESTLEY, 1970) the growth ratio is higher in the proximal part and then declines continuously toward the apex, thus a form of allometric growth is present. It is important to note this here because during the development of other appendages, such as legs, neither a marked allometric growth nor a specific proliferation zone has been described. Similar to this can be also the condition of, e.g., the part of insect antennae produced during embryogenesis. In the grasshopper antennae the mechanism of article production during postembryonic development involves the division of the meristematic article (called meriston) and of just few articles close to it (CHAPMAN & GREENWOOD, 1986; CHAPMAN, 2002). During embryonic development, however, at least six articles appear simultaneously and mitoses were found all along the antenna (CHAPMAN, 1970).

A growth zone in the flagellar structures of arthropod appendages involves thus three different, but clearly connected, aspects: lengthening, production of new articles and cellular proliferation. A growth zone is a more or less restricted zone where these three processes are located during postembryonic development. How cellular division, production of new articles and lengthening (and cell shape change can also play an important role in this respect, see below) are connected to each other is not clear yet.

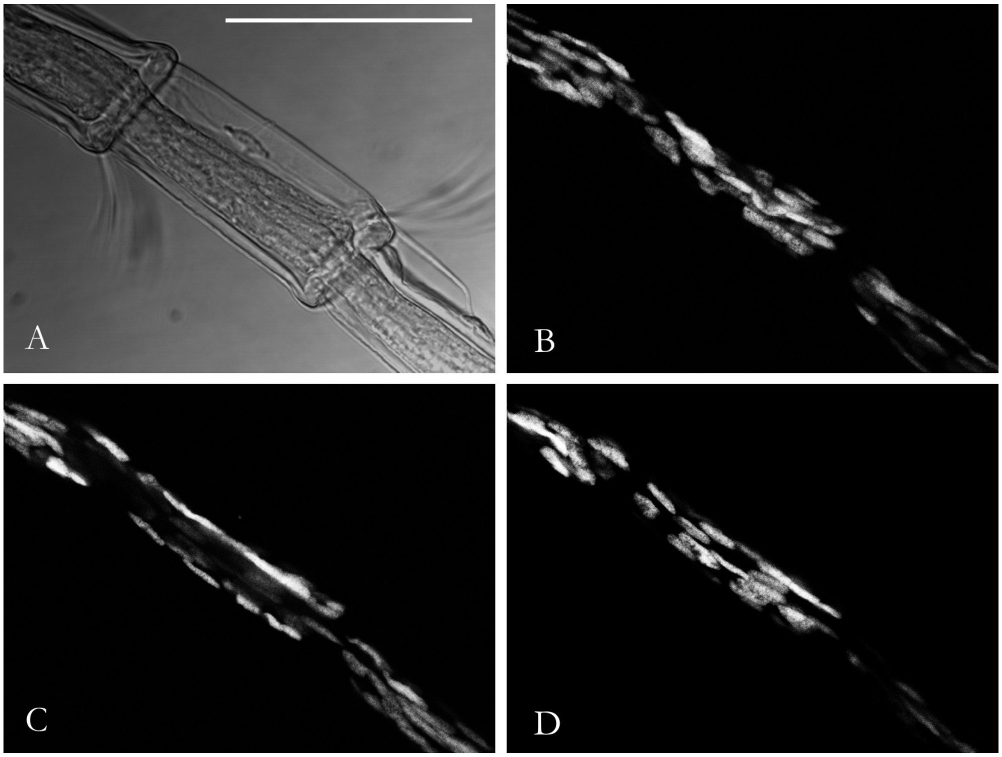

Fig. 7.8. Article in the distal part of the antennal flagellum of *Asellus aquaticus*, external morphology and nuclear staining. A bright field view (A) and three single slides of a CLSM stack (B, C and D) at different focus levels are shown. Scale bar 50 µm.

EPIDERMAL CELL SHAPE CHANGE DURING ARTHROPOD APPENDAGE DEVELOPMENT

In his review on the crustacean epidermis, FREEMAN (1993) named “larval cells” those epidermal cells actively cycling (and with rapid replication), generally of cuboidal shape and with low cytoplasm to nuclear volume ratio. He also noted that cells involved in replication are often found in specific locations. In contrast to the larval epidermal cells, FREEMAN (1993) identified several different kinds of differentiation through which the epidermal cells can go. Among these he named “general epidermal cells (GECs)” those cells that differentiated changing their shape, acquiring a greater apical surface and, mostly, not cycling anymore. He later showed (FREEMAN, 2005) that the differentiation to GECs (thus the expansion of the apical surface of the individual epidermal cells by shape change) contributed significantly to the linear growth of the branchiopod phyllopodal limbs.

I have shown here that, in both models studied, cells are produced in a rather specific site and are then moved away from this site by the production of new cells. Meanwhile cells change their shape, the nuclei passing from a rounded to a thin and long shape. This model clearly aligns with the presence of a specific proximal position of larval cells (as defined above) that produce cells that will later become differentiated into GECs changing their shape and not dividing anymore.

I have noted above that the flagellar structures show a marked allometric growth correspondent to the growth zone. Cells division, thus, seems to play the most important role for the linear growth of these parts of appendages. However, the extent to which differentiation into GECs contributes to the linear growth and the eventual presence of programmed cell death (which may also be an important process in epithelial morphogenesis; FRISTROM, 1988) is not known.

GROWTH ZONE IN ARTHROPOD APPENDAGES

MARUZZO *et al.* (2007) discussed the developmental similarities between the antennal flagellum of malacostracans (for both their first and second antennae) and insects in the production of new articles during postembryonic development. There is one article (usually the first of the flagellum) which is the producer of new articles and, eventually, there are some further divisions of the articles produced by it (see also MARUZZO, 2008a for a more specific discussion on isopod second antennal flagellum). Their discussion needs to be expanded here.

Although data are very scattered, the presence of a proximal growth zone in the antennal flagellum of malacostracans and insects appears rather conserved. A growth zone, however, have been shown here not to be just restricted to the antennal flagellum, but to be shared also by another structures, namely the rami of amphipod pleopods and I guess that a similar growth zone is present in other appendages too (see FERRARI & DAHMS, 2007 for a comparable growth zone in different copepod appendages). In addition, I have shown that the similarity at the cellular level does not involve only cellular proliferation, but also similar differentiation into GECs. While this aspect has not been considered in other studies, I guess that a corresponding similarity can be found in other similar structures too.

MARUZZO *et al.* (2007) restricted the similarity in antennal flagellum development to malacostracan crustaceans and insects, because they found no support for a growth zone in the antennae of "entomostracan" crustaceans and myriapods. They obviously argued that this similarity of malacostracans and insects could thus be due to a close phylogenetic relationship or to convergent evolution. However, a more accurate bibliographic search showed that a proximal growth zone has also been described for the antennae of some myriapods. Millipedes have antennae composed of few articles, usually 8 in the adults, but they usually add few articles during postembryonic development. *Polyxenus lagurus* (Linnaeus, 1758) is not an exception, at hatching it has 5 articles and as adult it has 8; this increasing is accomplished by the division of the first antennal article and the article immediately close to it (NGUYEN DUY-JACQUEMIN, 1972). Symphylans have long antennae composed of many articles, most of these articles are produced during postembryonic development by the division of the first antennal article only (TIEGS, 1945; see also MICHELBACHER, 1938). TIEGS (1945) also described the epidermis of this basal article as the main centre of mitoses.

MARUZZO *et al.* (2007) did not include myriapods and "entomostracan" crustaceans mainly because lack of detection of a specific growth zone in the antennae of centipedes and in the first antennae of copepods and ostracods. Concerning centipedes they based their observation on MINELLI *et al.* (2000) which, for antennae that gets new articles during postembryonic development, is mainly based on the ontogenetic observation of SCHEFFEL (1969) on *Lithobius forficatus* (Linnaeus, 1758); this species, however, adds new articles in the antennae during the whole life, while SCHEFFEL (1969) described only the first postembryonic instars. Since while the appendage is relatively short and composed of few articles a specific growth zone can be overlooked, especially if the growth zone is not restricted to the first article but it is also spread to several articles close to it (as it happens, e.g., in *Asellus*), only specific observation on late postembryonic development can clarify if, in this model, a specific growth zone is lacking. Ostracod first antennae are always relatively short and with few articles (see discussion and further references in SMITH & TSUKAGOSHI, 2005 for the postembryonic development of the first antennae), so if a growth zone exists but it is not restricted to an article, it would be hard to detect. Copepods, anyway, can also have relatively long first antennae with many articles but, according to the model of BOXSHALL & HUYS (1998), no specific growth zone can be found; studies on some copepod groups, however, identified one (for Harpacticoida; DAHMS, 1989) or three (for Calanoida; FERRARI & BENFORADO, 1998) specific centre of production of new articles.

Since a growth zone seems phylogenetically widespread I here propose that a growth zone (as defined above) in the first antennae and in the rami of postantennulary appendages is a primitive condition for the postembryonic development in, at least, mandibulates arthropods (myriapods, insects and crustaceans). Derived condition where such growth zone is not found, as it may be the case of centipede antennae and copepods first antennae, may well be present anyway. Concerning the proximal part (protopod) of crustacean postantennulary appendages, recently FERRARI & DAHMS (2007) provided convincing arguments for the presence of a growth zone (intended by them just as a site where new segmental structures are produced) proximally, where the appendage meets the body wall.

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DISCUSSION AND CONCLUSION

RELATIONSHIP BETWEEN MUSCLES, MUSCLE INSERTIONS AND JOINTS

Evidence showing that the development of joints, muscles, and muscle insertions are developmentally correlated processes in the insect legs has been summarized in the introduction (pp. 21-22). In this thesis I showed (MARUZZO, 2008b, 2008c) that this is likely true also for other arthropod appendages.

Following BOXSHALL's (2004) definitions of annuli and true articles, it is possible to identify four kinds of joints: those with muscle insertion(s) (thus, joints of a true article), those without muscle insertion but with muscle(s) passing through (joints of an intercalary annulation), those without muscle insertion but with tendon(s) passing through (joints of an intercalary annulation), and those without muscle insertion and with no muscle or tendon passing through (joints of a terminal annulation). Joints without any muscle insertion can occur, but these have either no muscle passing through or just tendons, the occurrence of joints without any muscle insertion but with muscle(s) passing through is very doubtful (MARUZZO, 2008c). Joints without any muscle insertion and with no muscle passing through are present in relatively few kind of arthropod appendages and in groups that may be phylogenetically close related (MARUZZO, 2008c); thus, it is likely that this kind of joints emerged few times, or even one, in arthropod evolution.

The possible non-existence of joints without muscle insertions but with muscles passing through, clearly suggest the presence of developmental constraints: when a joint is produced close to a muscle, the muscle must get an insertion (or vice versa). In other words, when some epidermal cells differentiate into arthrodial membrane cells close to a muscle, some epidermal cells differentiate also into tendinal cells (or vice versa). Other evidence of a developmental constraint derives from the antennal exopod of *Artemia* nauplii. In this model there are not complete joints, but just "partial" cuticular folds; this is probably a derived condition and each of these folds have its muscle insertion (MARUZZO *et al.*, 2008). Much has been written about the reciprocal signalling between epidermis and muscles during *Drosophila* trunk development (review in, e.g., VOLK, 1999), but many things are still unclear. However, it is interesting here to note that epidermal cells of *Drosophila* differentiate into tendinal cells if they get in contact with differentiated muscle cells *in vitro* (TUCKER *et al.*, 2004).

However, there are joints without muscle insertions but with tendons passing through. While the histological distinction between a muscle and a long tendon is rather clear, their development needs to be investigated further. There is currently little doubt that muscles have mesoderm origin. Arthropod muscle insertions are usually regarded as an ectodermal product, but this is certain only regarding tendon cells (connecting epidermal cells between muscle and cuticle). However, some muscle insertions can have more or less long tendons, or tendon matrix; these structures, together with others, have been collectively named "connective endoskeleton" by BITSCH & BITSCH (2002), and their developmental origin is still unclear.

To understand the origin of apomorphic characters of a group is usually useful to look at the closest relative of this group, which may have retained a condition more similar to their common ancestor than the group under study. For arthropods, onycophorans are among the closest relatives (e.g., MALLATT & GIRIBET, 2006). If we assume that the "circular folds" or "segment-like rings" in the onychophoran "unjointed legs" are somehow homologous to arthropod appendage joints, it is of interest to note that onychophoran appendage "circular folds" are apparently equipped with muscle insertions (SNODGRASS, 1935, 1938). This suggest that a close developmental relationship between muscle insertions and cuticular invaginations was already present before the emergence of arthropods and that, as already noted (e.g., WALOSZEK *et al.*, 2005), a subsequent evolutionary transition involved the production of sclerotic and softer cuticular areas in the appendages.

Among true articles, I noted developmental differences between articles equipped with muscles confined within that individual article (in most cases, a pair of antagonistic muscles) and articles which have just intermediate insertions of muscles that do not originate or end within that article (MARUZZO, 2008b). The evolutionary transition between these two, however, may involve nothing more than heterochronic changes during muscle development (MARUZZO, 2008c).

MECHANISM OF ANNULATIONS IN FLAGELLAR STRUCTURES

Flagellar structures in arthropod appendages often show remarkable similarities in their development (MARUZZO *et al.*, 2007; MARUZZO, 2008a, 2008d). "Flagellar", however, is a functional definition (usually applied to the terminal parts of an appendage performing whip-like movements by muscles inserted at its base), and it is not always appropriate to call annuli the articles that compose a flagellar structure (MARUZZO, 2008b, 2008c). The developmental similarities of such apparently different things, however, can be explained by derivation from a common ancestor. Although data are very scattered, their similarities can be related to the presence of a "growth zone", which I have proposed to be an ancestral condition for the postembryonic development of the (first) antennae and rami of postantennulary appendages of, at least, mandibulate arthropods (MARUZZO, 2008d).

From a developmental point of view, this growth zone is not only where new articles are produced (during both normal development and regeneration; MARUZZO *et al.*, 2007, in press; MARUZZO, 2008a), but also where mitoses are localized (MARUZZO, 2008d). The presence of such growth zone where both patterning (production of periodically arranged structures) and growth (cellular proliferation) are localized strongly suggests that these two processes, whose mutual relationship is not clear for the arthropod trunk, are at least highly coordinated the postembryonic development of arthropod appendages.

ANNULI AND TRUE ARTICLES

Which are then, if any, the developmental similarities between the annuli of different appendages of the same species and between homologous appendages of different arthropod species? And which are, if any, the real developmental differences between annuli and true articles? If we defined annuli by the presence/absence of intrinsic musculature, we must acknowledge that 1) there are developmental similarities between annuli of different appendages and different arthropods, but these are not peculiar to annuli, and 2) there is no clear developmental difference between annuli and true articles.

More valuable considerations, however, can be done if we consider as annuli (as it is often done, irrespectively of the muscular system) the articles that can not move the appendage with some independence from other articles. In these case, there are some developmental similarities between annuli of different appendages within a species and between different arthropods; but these similarities are related to the presence of a growth zone which 1) is found only in postembryonic development, and 2) is derived from a common ancestor (see above). There are also developmental differences (timing of appearance) between these articles, but these differences are the results of functional constraints.

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