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Segmental mismatch in crustacean appendages: The naupliar antennal exopod of *Artemia* (Crustacea, Branchiopoda, Anostraca)

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ABSTRACT

Based on traditional techniques and confocal laser scanning microscopy for external morphology, and immunohistochemistry for the muscular system, we describe here the segmental features of the antennal exopod of *Artemia* nauplii. Two kinds of serial elements are present, i.e. setae (with cuticular folds at their base) and ringlets (serially arranged sclerites separated by joint-like cuticular folds not extending to form complete rings around the appendage). The two series are usually not in register. The cuticular folds of the setae and of the ringlets are also sites of intermediate insertions of the three exopod muscles: as the two tegumentary structures are discordant in periodicity, this is also mirrored in the pattern of muscle insertions on the two sides of the appendage. Similar cases of segmental mismatch are known for the trunk of several arthropods, but segmental mismatch along the appendages has received very little attention. The occurrence of segmental mismatch in the naupliar appendages of both extant and fossil crustaceans is reviewed and it is suggested here to be a primitive feature of the exopods of both second antennae and mandibles. Problems in the interpretation of morphological evidence are discussed, also in relation to development and evolution of segmentation of naupliar appendages.

1. Introduction

A segmental pattern can be defined as the serial occurrence of homologous structures along one axis, either the main body axis or the longitudinal axis of an appendage. However, there are cases where the segmental series of different repeating (periodic) structures along the same axis show discordant arrangement. This condition is commonly termed 'segmental mismatch'. Segmental mismatch in the trunk of arthropods is not so rare. The most celebrated case is that of the notostracan crustaceans, with their marked differences in periodicity, length of the series and postembryonic segmentation schedule among dorsal and ventral structures (Linder, 1952). Very numerous and diverse cases of mismatch of trunk features are also present in myriapods (see Fusco, 2005). Segmental mismatch along the appendages has received comparably little attention, but this condition is conspicuous in the head appendages of many crustaceans, as we discuss in this paper.

Crustacean nauplii are characterized by having three pairs of functional appendages: first antennae (or antennules), second antennae, and mandibles. The naupliar phase is usually divided into orthonauplius, with only the naupliar appendages present, and metanauplius, 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 part (the protopod) bearing an outer exopod and an inner endopod, the first usually longer than the latter. Both rami are generally described as "multisegmented" or "multiannulated". This is the case also for the antennal exopod of several anostracan nauplii (e.g., Fryer, 1983; Møller et al., 2004; Olesen, 2004). However, from available descriptions of the antennae of *Artemia* nauplii, the segmental pattern is unclear. Some authors are silent about the segmental condition 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. For example, of the antennal exopod of cephalocarids is divided into well-defined articles and has muscles running parallel to the proximo-distal axis, some of them extending to the last joint. Usually, muscles have an intermediate insertion at each site of articulation they pass through (Hessler, 1964). The musculature of the naupliar antennal exopod of *Artemia* has been described only partially (Benesch, 1969; Kiernan and Hertzler, 2006; see also Fryer, 1983 for another anostracan, *Branchinecta ferox*). Three muscles run throughout the exopod, one dorsal and two ventral. None of these works provids detailed information on muscle insertions, although other authors briefly mention their existence (e.g., MacRae et al., 1991; Criel and MacRae, 2002).

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Here we describe the external morphology and the musculature of the antennal exopod of the nauplii of *Artemia* sp.; we show that it has two different series of segmental structures which are usually not matching. The segmental mismatch observed in this appendage along with other similar cases described in extant and fossil crustaceans and stem-crustaceans are discussed, both from an evolutionary and developmental point of view.

2. Materials and methods

Artemia nauplii freshly hatched from cysts obtained from a commercial supplier (INVE, Belgium) were fixed overnight at 4 °C in 4% paraformaldehyde in PBS, washed in PBS and eventually stored in PBS with 0.05% of sodium azide at 4 °C; alternatively, they were fixed and stored in 2% paraformaldehyde, 2.5% glutaraldehyde in PBS. Only orthonaupliar and early metanaupliar stages (less than 1 day old and with only three postmandibular appendage buds) 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 (5% in water, overnight at 50 °C) were washed in PBS with 0.3% Triton-X 100, eventually stained with chlorazol black, and mounted in glycerol. Observations were made with a Leica DM5000 B microscope using bright field light or differential interference contrast (DIC). In recent times 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 and 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 5–10 min, 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 fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in PBS were dehydrated in graded ethanol series, dried with hexamethyldisilazane (Sigma) (Nation, 1983), and coated with gold or dehydrated in graded acetone series, critical-point dried with carbon dioxide and coated with palladium-platinum. Examinations were made with a Cambridge Stereoscan 260, with a Jeol JSM-6490 or with a Jeol JSM-6335-F scanning electron microscope. Number of elements of segmental series were counted in digested specimens observed with bright field or differential interference contrast; specimens next to ecdysis (showing 'a cuticle inside a cuticle') were discarded, as counting and alignment of segmental structures was unreliable.

The musculature was investigated with phalloidin staining (which stains filamentous actin), while the pattern of muscle insertion was studied with both phalloidin staining and an antibody against α-tubulin (of which *Artemia* muscle insertions are known to be rich; Criel et al., 2005). Specimens fixed in 4% paraformaldehyde in PBS 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 1–2 h at room temperature. A primary antibody against α-tubulin made in mouse (Sigma) was used (1:750, overnight at 4 °C). After several washes in PBS, samples were incubated for 1 h 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 4 h 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) has performed as above but omitting the incubation with serum and antibodies. 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 tissues was present.

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

In describing *Artemia* antennal morphology and anatomy, we avoid terms like 'antennal article', 'antennal segment', or 'antennomere'. These terms imply that the appendage is comprised of a series of functional units of articulation, with sclerite rings alternating with close belts of arthrodial membrane. This does not apply in general to appendages affected by segmental mismatch. Consistently, we use the term 'joint' to indicate a site of articulation, irrespectively of whether it is a close loop around the appendage or an incomplete transversal fold (incomplete loop).

3. Results

3.1. External morphology

The external morphology of the antennal exopod of *Artemia* nauplii is shown in Fig. 1. On the posterior-ventral side there are long natatory setae. In our sample ($n = 119$), these setae are in the number of 8–11 plus a small apical one. Each seta, to the exclusion of the apical one, is inserted on a smooth transversal ridge that distally presents a cuticular fold resembling a joint ('setal fold'). On the anterior side of the exopod there is a proximo-distal series of 'ringlets'. We call ringlet an element of a series of sclerites with no evident cuticular thickening, separated from the contiguous ones by small joint-like cuticular folds ('ringlet folds'), which do not produce a complete ring around the appendage. Ringlets present a row of denticles (short spines) on their distal margin. Denticles are usually present (although not arranged in rows) also in other parts of the appendages and on the trunk. In our sample, the number of ringlets per exopod ranges from 9 to 14. The ringlets are in direct contact with the base of the setae on the ventral side, but not on the posterior-dorsal side; on the latter side there is a zone of soft cuticle (which, following preparation for SEM, usually shrinks more easily than other parts of the exopod) with no denticles and where usually the setal folds extend (Fig. 2).

Variation in the number of setae and ringlets does not seem to be due to postembryonic increase since no significant difference was found between orthonauplii and metanauplii (Table 1; mean difference one-tailed t-test, d.f. $= 117$, $p > 0.10$).

Although both setae and ringlets show a well defined serial arrangement, in most specimens the two kinds of elements are not in register, a clear case of segmental mismatch (Figs. 1 and 3; Table 1).

3.2. The muscular system

As already noted in *Artemia* (Benesch, 1969; Kiernan and Hertzler, 2006) and in *Branchinecta ferox* (Fryer, 1983), three muscles run throughout the antennal exopod: two on the side of the natatory setae, one on the side of the ringlets. All these muscles have several intermediate insertions along their course (Fig. 4). Muscle insertions are thin strings rich in both actin (Fig. 4) and α-tubulin (Fig. 5). For the two muscles on the posterior-ventral side, these strings (tendons) attach to the cuticle on each setal fold, while the tendons of the single muscle on the anterior side has insertions on each ringlet fold (Fig. 6). Thus, the mismatch between setae and ringlets is also reflected in the arrangement of muscle insertions

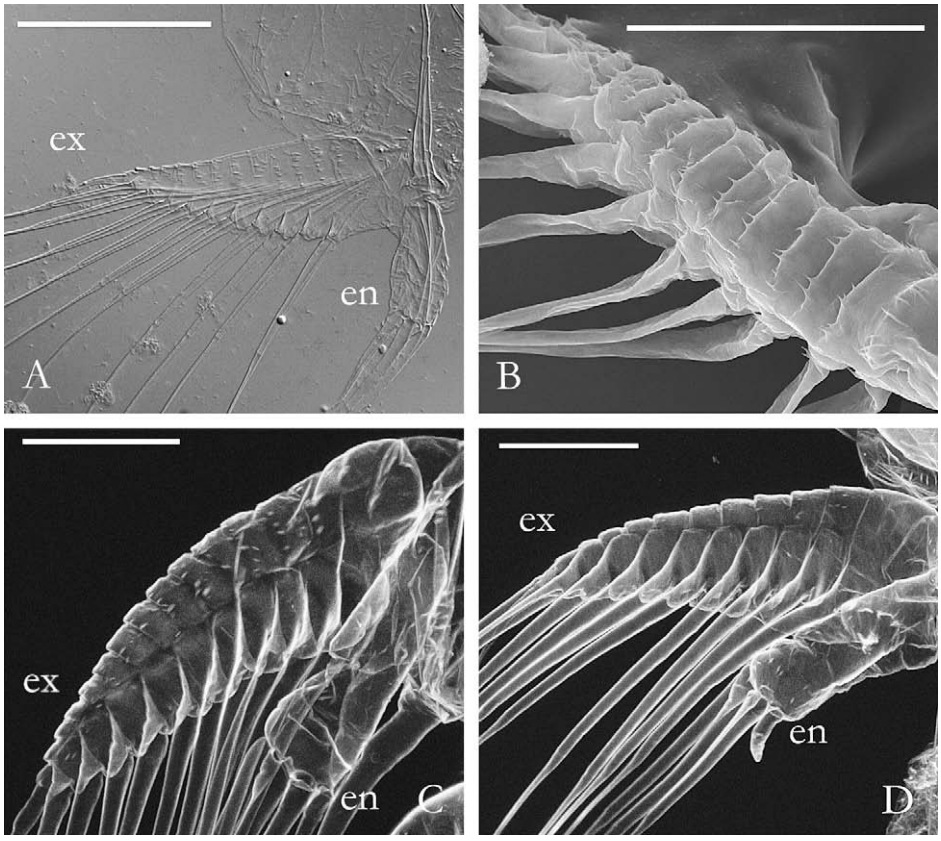

Fig. 1. The external morphology of the second antenna of a nauplius of *Artemia*. (A) DIC; scale bar 100 μm. Nervous system (the hardest tissue to be digested) extending into the setae is also visible. (B) SEM, anterior-ventral view; scale bar 50 μm. (C, D) Maximum intensity projections of stacks of pictures obtained with CLSM; scale bars 50 μm. Note that the number of natatory setae on the posterior-ventral side does not match with the number of ringlets on the anterior side in comparable extents of the appendage. en, endopod; ex, exopod.

(Figs. 4 and 6). The terminal (distal) insertion of posterior-ventral and anterior-dorsal muscles is at the distalmost setal fold or at the distalmost ringlet fold, respectively (Fig. 6).

4. Discussion

When a segmental pattern is referred to a whole axis, either the main body axis or the axis of an appendage, rather than to a specific set of structures serially repeated along it, the idea of a body or a limb 'comprised of' unequivocally defined blocks (trunk segments, appendage articles) will result (Budd, 2001). 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 difficulties arise in describing cases of segmental mismatch, when different serial structures along the same axis show discordant serial arrangement.

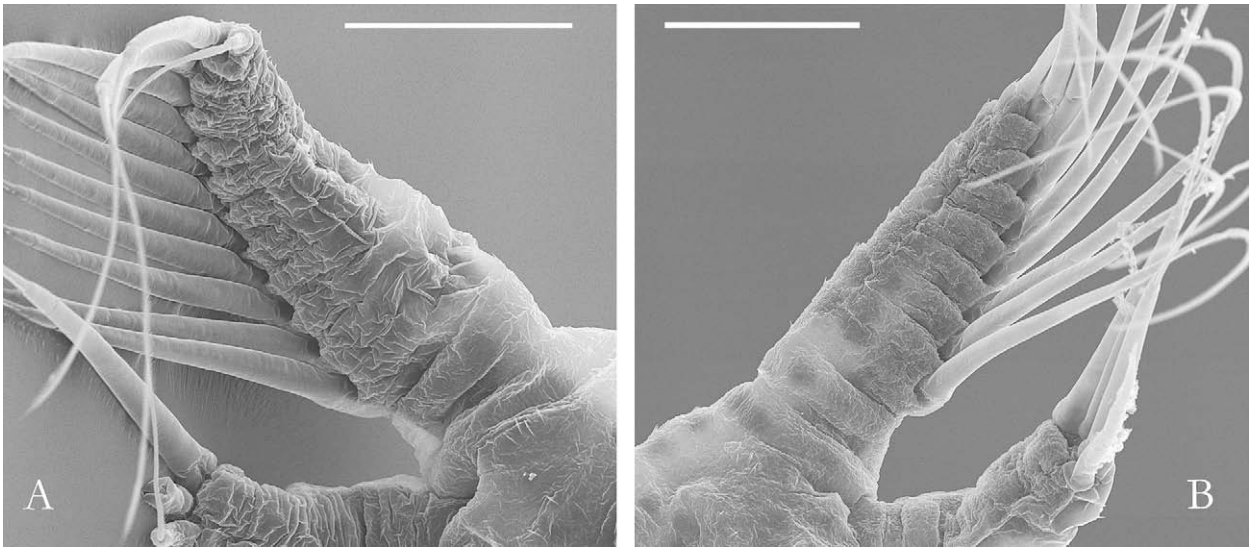

Fig. 2. Posterior side of the naupliar second antennal exopod of *Artemia* showing the setae and the zone of soft cuticle posterior to them. Two different specimens are shown (A and B). Scale bars 50 μm.

Table 1
Number of setae and ringlets in the antennal exopod of *Artemia* orthonauplii and metanauplii

Number of setae	Number of ringlets	Number of observed cases	
		Orthonaupliar exopod ($N = 56$)	Metanaupliar exopod ($N = 63$)
8	10	1	2
8	11	5	–
9	9	1	–
9	10	7	6
9	11	10	12
9	12	8	17
9	13	5	6
9	14	–	1
10	10	1	–
10	11	7	8
10	12	8	7
10	13	3	2
11	11	–	2

The numbers of setae do not include the small apical seta.

The antennal exopod of *Artemia* nauplii described here exhibits evident segmental mismatch. At the level of external morphology and muscular anatomy it is possible to identify two distinct segmental series, the setae and the ringlets. However, only rarely do the two series match (in our sample, in four cases out of 119, i.e., about 3%). In the following sections we provide a review of similar segmental mismatch in several crustaceans and stem-crustaceans. In describing the exopod of *Artemia* naupliar antennae we adopted four terms: setae, setal folds, ringlets, and ringlet folds. The term 'ringlet' has already being used in describing similar structures in 'Orsten' fossils (e.g., Müller and Walossek, 1985, 1988; Walossek, 1993). The terminology used by different authors for similar structures in other branchiopods (and in other crustaceans as well) is far from uniform. Irrespective of the actual terms used by different authors, whenever possible we use in the following the terminology adopted for *Artemia* in the above section.

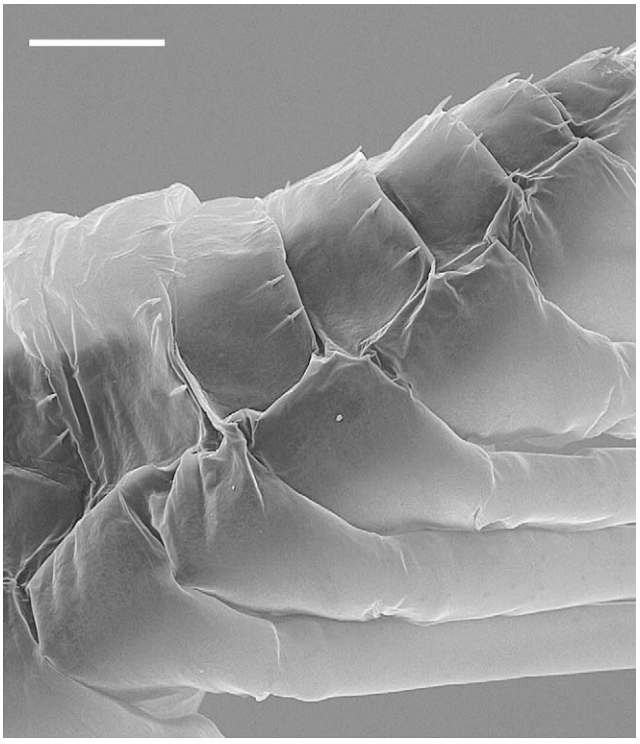

Fig. 3. Mismatch between setae and ringlets in the naupliar antennal exopod of *Artemia* (anterior view). Scale bar 10 μm.

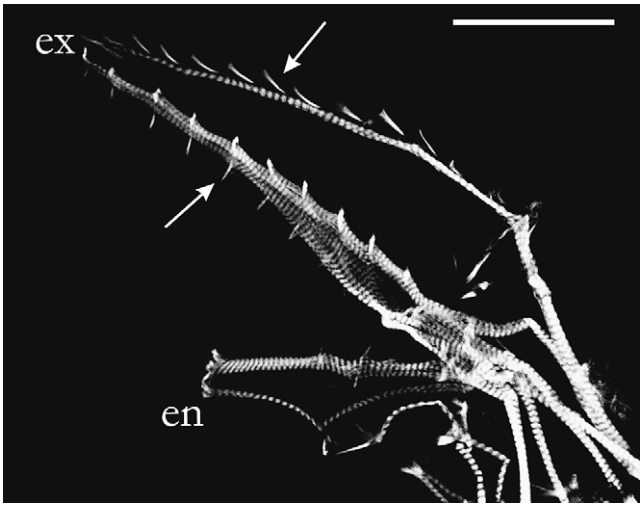

Fig. 4. Musculature of the second antenna of a nauplius of *Artemia* (maximum intensity projection of a stack of pictures obtained with CLSM, phalloidin staining). Arrows point to two intermediate muscle insertions (among many). en, endopod; ex, exopod. Scale bar 50 μm.

4.1. Segmental mismatch in crustacean appendages

The exopod of the second antennae of branchiopod nauplii is generally described as multisegmented or multiannulated. For instance, in the nauplii of the anostracan branchiopod *Eubranchipus grubii*, the appendage presents one seta on each "annulation" (Møller et al., 2004), i.e., the series of setae and ringlets are in register along the exopod. A well-segmented antennal exopod is present in the nauplius of the laevicaudatan *Lynceus brachyurus* (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 is described as having unclear segmentation, revealed by "rows of minute spines" (=denticles) and "articulation-like constrictions" (=ringlet folds) (Møller et al., 2004; see their Figs. 4C and 7C). A comparable morphology is found in the antennal endopod of other branchiopods (in *Artemia*, Schrehardt, 1987, see his Fig. 5; in *Eulimnadia*, Olesen and Grygier, 2003, see their Fig. 5D; in *Limnadopsis*, Pabst and Richter, 2004, see their Fig. 2H).

Among other extant branchiopods, spinicaudatans exhibit an interesting feature. During the postembryonic development of *Caenestheriella gifuensis*, the 'segmentation' of both the antennal exopod and endopod gets reduced. This reduction, however, involves only the ringlets, but not the few setae present along the exopod (the endopod has setae only at the tip) (Olesen and Grygier, 2004; see, e.g., their Fig. 8B).

In the Upper Cambrian *Rehbachiella kinnekullensis* (a fossil crustacean with branchiopod affinities; e.g., Møller et al., 2004; Olesen, 2004), the proximal part of the first antennae was made of ringlets ("incomplete annuli"), whose number increased slightly during postembryonic development, while the posterior side was not segmented (Walossek, 1993). In the exopod of both second antennae and mandibles the setae might (Walossek, 1993; see Fig. 3 of his plate 19 for an antennal exopod) or might 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 ringlets. Concordance or mismatch between setae and ringlets of these appendages is, specifically, one of the features used by Walossek (1993) to distinguish two possible alternative ontogenetic pathways (or "larval series") in *R. kinnekullensis*.

"Several partial rings of minute teeth" (i.e., ringlets) were also described (Scourfield, 1940) for the larval stage of the Devonian

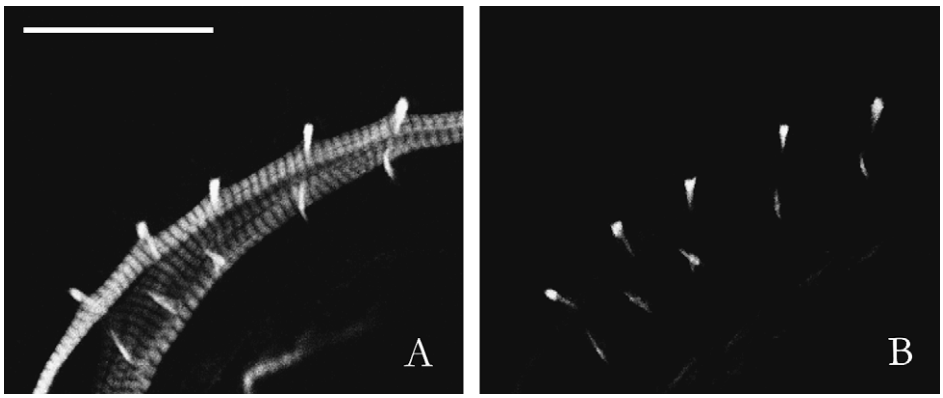

Fig. 5. Muscles and their insertions in the antennal exopod of a nauplius of *Artemia* (maximum intensity projection of a stack of pictures obtained with CLSM). The same portion of the appendage is shown, at the same magnification, to visualize both actin (A; phalloidin staining) and α-tubulin (B; antibody against α-tubulin staining). Scale bar 30 μm.

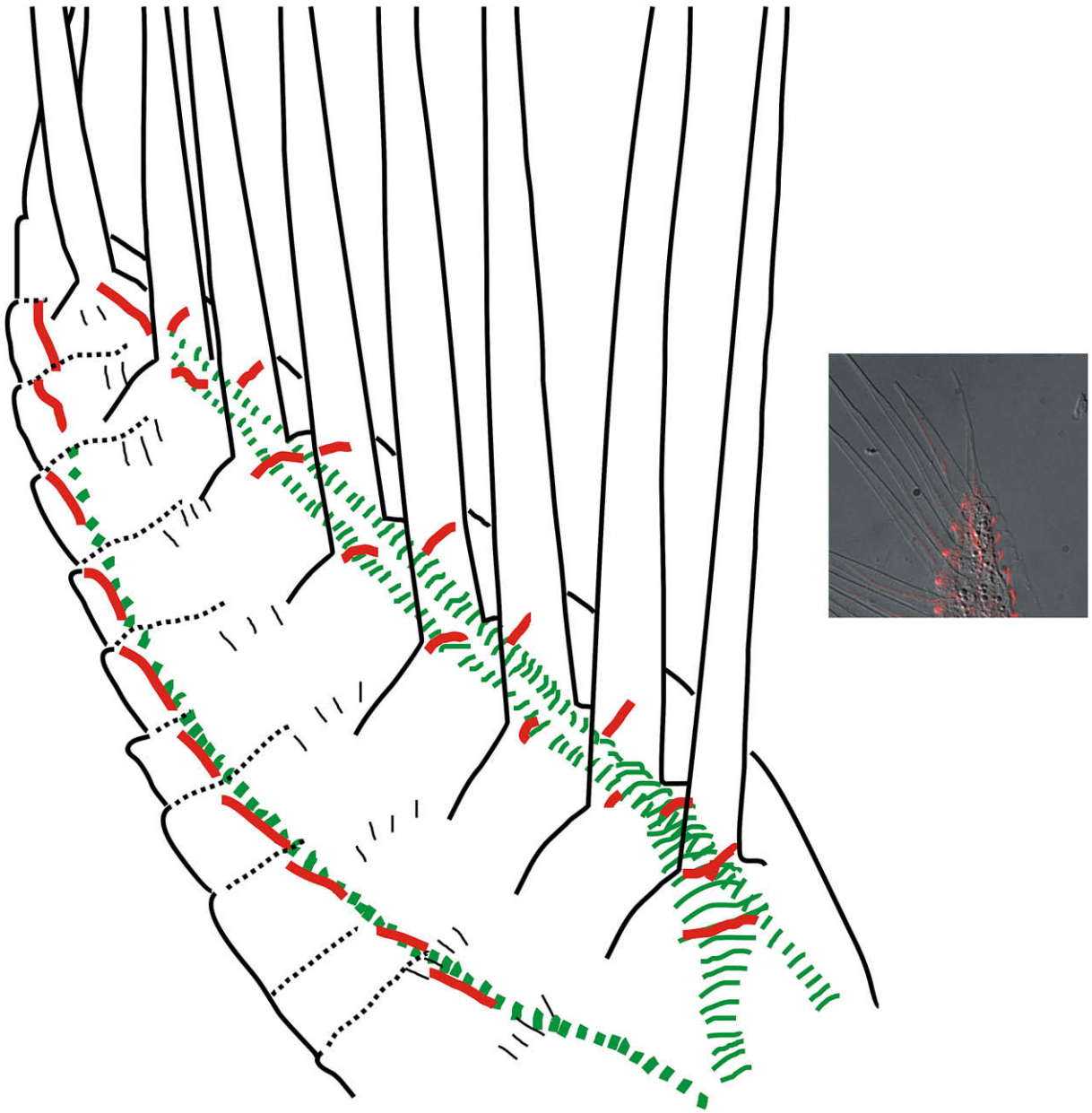

Fig. 6. Schematic drawing of the naupliar antennal exopod of *Artemia*. Cuticle is in black, muscles in green and muscle insertions in red. The drawing, based on one specimen, was made digitally from different focus-level photographs as described in Section 2 for studying the location of muscle insertions. Inset shows an overlay of photographs obtained with DIC and red epifluorescence filter (antibody against α-tubulin staining).

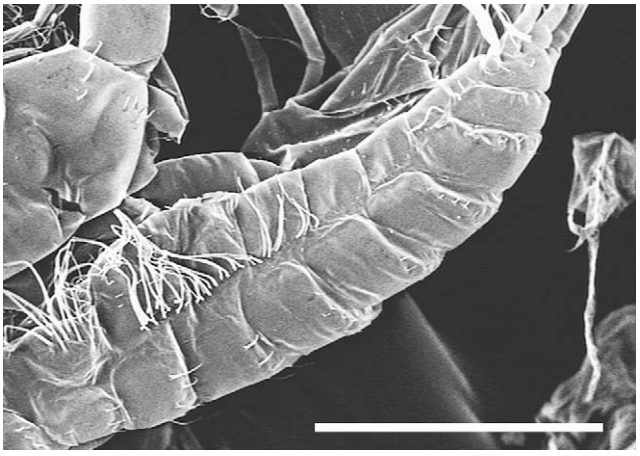

Fig. 7. Antennal naupliar exopod of *Balanus improvisus*. Dorsal view, SEM; the slipped ringlets and the longitudinal groove are shown. Scale bar 50 μm.

Lepidocaris rhyniensis (possibly, a stem lineage anostracan; Møller et al., 2004).

In the Upper Cambrian *Bredocaris admirabilis* (a fossil crustacean with possible thecostracan affinities; Müller and Walossek, 1988; but see Boxshall, 1998 for discussion on possible branchiopod affinities) the first antennae were composed of three "articles", but the most proximal one, which was much longer than the others, was further divided by short ringlets limited to the anterior side. Apparently, the number of ringlets increased slightly during postembryonic development (Müller and Walossek, 1988). The exopod of the second antennae was also divided into ringlets on the outer side. On the inner side there were natatory setae which did not exactly match with the ringlets (Müller and 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 and Walossek, 1988; see especially Fig. 6 of their plate 4).

In the Cambrian phosphatocopines (traditionally classified with ostracod crustaceans but currently regarded as the sister group of Eucrustacea, the group containing all extant crustaceans; see Maas et al., 2003; Maas and Waloszek, 2005) the first antennae have been described for several species as composed of "irregularly arranged but weakly defined annuli" whose number apparently increased during postembryonic development (Maas et al., 2003; see especially their Fig. 19). In *Hesslandona unisulcata* at least, the exopod of both second antennae and mandibles acquired new "articles" during postembryonic development; these serially repeated elements were described as "incomplete sclerotic rings" (i.e., ringlets) medially with a membranous area on which a seta usually inserts (Maas et al., 2003; see their Fig. 17). Mismatch between setae and ringlets has also been recorded (Maas et al., 2003; see their plate 27B).

In the extant rhizocephalan *Briarosaccus tenellus*, the "annuli" of the naupliar exopod of both second antennae and mandibles are "incomplete" and "interlocking" (Walossek et al., 1996; see their Figs. 8C, 12G and 21C). However, in this case, the 'mismatch' is not one between the segmental series of two distinct structures, but it consists of a single series of ringlets, fully encircling the appendage from one side to the other, but 'closing imperfectly' on the side opposite to the setae, with a slight sliding between the two ends. In the appendage with these 'slipped ringlets', the cuticular folds do not form complete loops around the appendage. Viewed from opposite sides, the appendage shows different symmetry: reflection (bilateral symmetry) on one side, and glided reflection (reflection combined with translation) on the other side. Identical morphology is also found in the naupliar exopod of second antennae and mandibles of another cirripede, the barnacle *Balanus improvisus* (personal observations, see Fig. 7).

In the Upper Cambrian *Skara* (a fossil crustacean with unclear affinities) the segmentation of the exopod of both second antennae and mandibles is not regular, since "on the posterior side there are more joints than on the anterior one" (Müller and Walossek, 1985). According to the original figures (Müller and Walossek, 1985; see Figs. 4 and 8 of their plate 7) this mismatch is due to slipped ringlets, as in *Briarosaccus*. Ringlets have also been described for the outer surface of the proximal part of the first antennae (Müller and Walossek, 1985).

It is thus clear that the segmental mismatch described here for *Artemia* is not an isolated case in crustaceans. Although for crustacean nauplii such as those of cephalocarids or copepods, similar mismatch have not been recorded, these fine morphological details

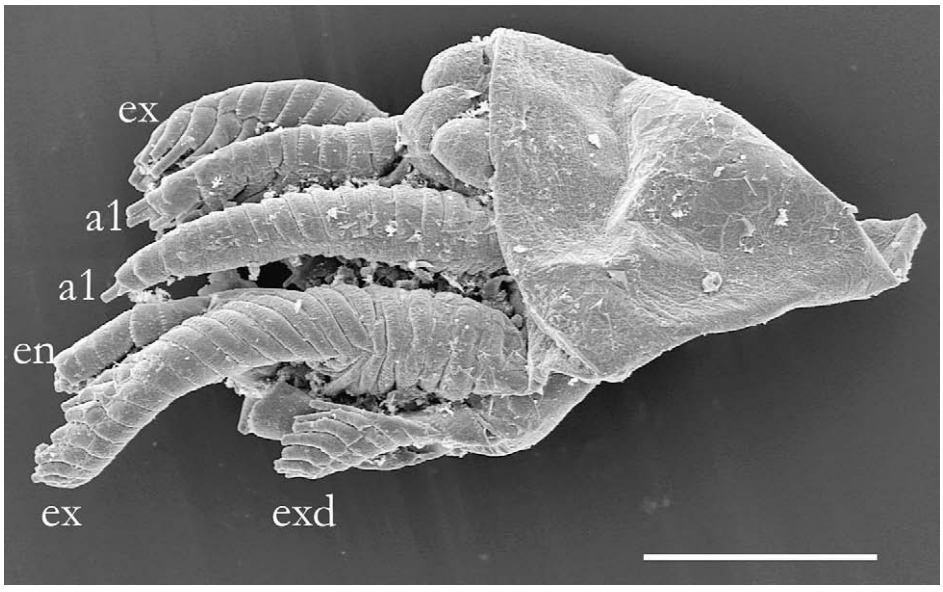

Fig. 8. Late stage (adult?) *Bredocaris admirabilis*. Ringlets are present only on the anterior side of the more proximal article of the first antennae (a1) and mismatch between setae and ringlets is present on the exopod of second antennae (ex). exd, exopod of the mandible; en, endopod of the second antenna. Scale bar 100 μm. Courtesy D. Waloszek (specimen UB W 276).

may have been well overlooked in taxa where these structures have not been explicitly looked for.

4.2. Segmental patterns and segmental artefacts

Walossek et al. (1996) considered the peculiar "annulated design" of *Briarosaccus* identical to that present in *Skara*, *Rehbachiella* and *Bredocaris*, and Maas et al. (2003) considered the mismatch found in phosphatocopines also identical to those of the previously mentioned species. While this may well be the case, caution should be used.

In *Briarosaccus* (and also in *Balanus improvisus*; personal observations) the mismatch of serial features along both the antennal and the mandibular exopod seems to be produced by slipped ringlets. On the side opposite to the setae, the slipped ringlets of cirripedes end in a longitudinal groove that runs all along the exopod, parallel to the proximo-distal axis (Fig. 7).

Similar longitudinal grooves have been indeed described for the previously mentioned fossils. Müller (1979) described such a groove in some species of phosphatocopines, although he did not report any segmental mismatch (mismatch later described by Maas et al. (2003), although they did not describe the grooves that are however evident in many figures of the paper). Fig. 1 of plate 11 in Walossek (1993) (a picture not mentioned by Walossek when discussing segmental mismatch in the exopod) shows the antennal exopod of a postlarval stage of *Rehbachiella* with somehow slipped ringlets ending on a longitudinal groove. Fig. 3D of Müller (1983) (and see also Figs. 4 and 5 of plate 3, Fig. 1 of plate 4 and Fig. 3 of plate 7 of Müller and Walossek, 1988) shows a longitudinal groove also in some naupliar appendages of *Bredocaris*.

However, somehow similar longitudinal grooves have been noted also in other fossils and in other appendages, where no segmental mismatch was reported. As discussed by Waloszek et al. (2005), the "line of connection" running along the endopod of trunk appendages of *Chengjiangocaris longiformis* (a Cambrian non-crustacean arthropod; Waloszek et al., 2005), as apparent from Fig. 16.5C of Hou et al. (2004) (and see also Figs. 13 and 14 in Hou and Bergström, 1997 and Fig. 4 of plate 1 in Budd, 2008), is similar to the just mentioned grooves of the antennal exopod of several fossil and extant crustaceans. A comparable longitudinal groove is even present in the rami of the two "post-cheliceral" appendages of the Upper Cambrian *Cambropycnogon klausmuelleri* (see Figs. 11C,F of Müller and Walossek, 1986; Text-Figs. 1, 5 and 6 of plate 3 of Waloszek and Dunlop, 2002) which, according to Waloszek and Dunlop (2002), is a pycnogonid (but see Bamber, 2007 for a different opinion).

In *Rehbachiella*, *Bredocaris*, phosphatocopines and *Artemia* the mismatch is due to a relative independence of the opposite sides, where different serial structures have different number and position (see Fig. 8 for an example of *Bredocaris*). Conditions in cirripedes may be different, as something like this has never been described thus far. While the presence of the longitudinal groove in cirripedes is out of question (in *Balanus improvisus* this groove is even marked by hairs, Fig. 7), this can be clearly observed only in some of the figured appendages of *Rehbachiella*, *Bredocaris* and phosphatocopines (see above), but not in others. Indeed, the most accurate description of an antennal exopod, in phosphatocopines, did not mention the presence of a longitudinal groove (Maas et al., 2003: 25), although, as mentioned, this feature is indeed observed sometimes. We can hypothesize that in these fossils, the longitudinal groove is an artefact of the fossilization process. Strongly in support of this hypothesis is the observation that, following preparation for SEM (see Section 2), most antennal exopods of *Artemia* nauplii showed a shrinkage or some kind of distortion more or less corresponding in position to the longitudinal groove (Fig. 9).

The condition of *Skara* requires a few more words. According to published evidence (Figs. 4 and 8 of plate 7 in Müller and Walossek, 1985), the exopods of both second antennae and mandibles seem to have slipped ringlets as in cirripedes. A mismatch in terms of number and position of serial structures is not evident in the best pictures available for the exopods (e.g., Fig. 2 of plate 7 and Fig. 1 of plate 15 in Müller and Walossek, 1985), although the statement that "on the posterior side there are more joints than on the anterior one" (Müller and Walossek, 1985: 11) would suggest otherwise. Thus, conditions in *Skara* are probably closer to those in cirripedes (slipped ringlets and no mismatch in number and position of serial structures) than in *Rehbachiella*, *Bredocaris*, phosphatocopines and *Artemia* (mismatch in terms of number and position of setae and ringlets and no slipped ringlets; longitudinal grove sometimes present as an artefact).

In this respect, the zone of soft cuticle just posterior to the setae described here for *Artemia*, which has no denticles but usually presents posterior expansions of the setal folds (Fig. 2), suggests a cautionary approach to morphological comparisons of naupliar appendages across crustaceans. This zone has not been described in other fossils or extant crustaceans but it can be easily overlooked since setae are usually bent on the posterior-dorsal side (at least in *Artemia*). The overall morphology of the antennal exopod of *Artemia* nauplii (possibly very similar to conditions in *Rehbachiella*, *Bredocaris*, and phosphatocopines) may thus be not so different from that in cirripedes (and possibly *Skara*), the major differences being a heavier sclerotization of the zone posterior to

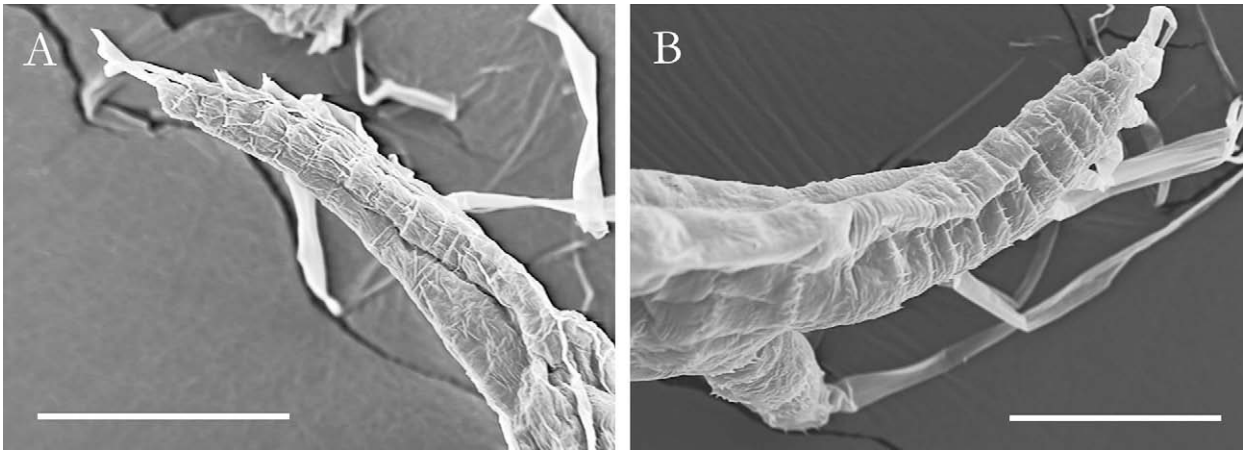

Fig. 9. Shrinked cuticle of the second antennal exopod of an *Artemia* nauplius resembling the longitudinal groove described for many fossils (dorsal views). Scale bars 50 μm.

the setae, independent from that of the anterior zone, and the presence of a longitudinal groove. If this interpretation is correct, a) the ringlets of *Artemia* correspond only to the anterior zone of the slipping ringlets of *Briarosaccus* (anterior and between the setae and the longitudinal groove) and b) the mismatch between setae and ringlets (as different number and position) must be considered a different thing. Apparently, setae and ringlets match well in the exopods of *Skara* (see above) and *Briarosaccus* Figs. (see 7D,E and 8C,D in Walossek et al., 1996) and this mismatch has never been described for other crustacean nauplii.

The presence of post-antennulary appendages with a multi-annulate exopod provided with one seta on each annulus was proposed as an apomorphic character for (Pan)crustacea (Walossek, 1999; Maas et al., 2003; Waloszek, 2003). While this may hold true for the postmandibular appendages (as far as we are aware, no comparable segmental mismatch has been described in appendages other than the naupliar ones), things may be different for the exopods of second antennae and mandibles, where the presence of ringlets and setae as not matching serial structures may be primitive. In fact, this character is found in many fossil and in extant Eucrustacea as well as in phosphatocopines. So, while the mismatch between setae and ringlets appears as a primitive feature of the exopod of the second antennae and mandibles of the nauplius, we must point out that there is variation in this feature. In the *Artemia* exopod we described here, about 3% of the recorded cases showed a good match between the two segmental series. Although our sample is too small for a quantitative evaluation of this percentage, cases like these are anyway expected on the basis of the independence of individual variation in the two series.

Summing up, different segmental conditions can be observed in the naupliar exopods of second antennae and mandibles:

1. perfect segmental match between the two series (e.g., *Rehbachiella* [in one of Walossek's (1993) larval series], *Eubranchipus*);
2. segmental mismatch between the two series (e.g., *Rehbachiella* [in the other larval series], *Bredocaris*, phosphatocopines, *Artemia*);
3. slipped ringlets but no mismatch in terms of number and position of serial structures (e.g., *Briarosaccus*, *Skara*);
4. complete rings dividing the exopod into articles (e.g., cephalocarids, ostracods, copepods, mystacocarids).

The distinction between the first and the fourth condition may be not so clear and it requires more specific observations on the presence and distribution of denticles and on the zone posterior to the setae. Anyway, a clear-cut distinction may not be present at all.

The appendage segmentation process may extend over a large portion of ontogeny, and in the face of superficial similarities the developmental origin of the mismatch could be different in different taxa.

Conditions in the first antennae and the endopod of second antennae and mandibles deserve further attention. We remarked on the presence of ringlets in the proximal part of the first antennae of *Rehbachiella*, *Lepidocaris*, *Bredocaris* (Fig. 8) and *Skara*. Walossek (1993) noted that these ringlets resemble the proximal subdivisions found in the first antennae of the nauplii of some extant malacostracans and thecostracans (see, e.g., Fielder et al., 1975; Kolbasov and Høeg, 2003). It must be noted, however, that from the available descriptions it is not clear if these subdivisions are produced by ringlets and if these have a distal row of denticles. The "irregularly arranged but weakly defined annuli" of phosphatocopines are not currently comparable with any eucrustacean.

While the naupliar endopod of second antennae and mandibles is usually described as well-divided into articles, we noted the possible presence of ringlets in the antennal endopod of different branchiopod crustaceans (*Eubranchipus*, *Artemia*, *Eulimnadia*, *Caenestheriella*, *Limnadopsis*), although in others (e.g., *Lynceus*, see Olesen, 2005) the endopod is apparently composed of articles.

Except for pointing out the probable primitive condition for the mismatch between setae and ringlets in the exopod of the second antennae and mandibles of the nauplius, we do not dare to advance further phylogenetic considerations since, as noted, the lack of specific observations for many groups could be highly misleading. Further observations will likely provide interesting data for phylogenetic discussion. In this respect it will be also interesting to get more precise observations on malacostracan nauplii (only present in euphausiids and in dendrobranchiate shrimps) which have likely evolved independently from the other crustacean nauplii (Scholtz, 2000).

4.3. Segmental mismatch in development and evolution

All the cases presented here show that different structures of the same naupliar appendage (e.g. setae and ringlets) can behave as independent segmental units. This implies some degree of independence of developmental pathways at the two sides of the appendage during embryogenesis (cf. Janssen et al., 2004 for independent dorsal vs. ventral segmentation in the trunk of the pill millipede *Glomeris*). However, postembryonic development of the antennal exopod and endopod of *Caenestheriella* involves changes only in the number of ringlets, not in the setae, showing that decoupled regulation of segmentation of the two sides can be maintained until late development. This may deserve comparison with the marked differences in segmentation schedule of the dorsal and ventral trunk structures in the notostracan *Triops* (Linder, 1952).

From a developmental point of view, this form of segmental mismatch, where the structures of opposite sides have different periodicity, shows that serial structures along an arthropod appendage can either use in different ways the same positional information available along the proximo-distal axis, or be regulated by different pre-patterns providing positional information. Quite a lot is known about the mechanisms patterning the proximo-distal axis of arthropod appendages (see reviews in Nagy and Williams, 2001; Angelini and Kaufman, 2005; Giorgianni and Patel, 2005; Prpic and Damen, 2008), but much less is known about the anterior-posterior and dorso-ventral axes (to the extent these are distinct), although some genes involved in the establishment of these axes, such as *H15* and *optomotor-blind*, have recently been identified and appear to have a conserved role (Janssen et al., 2008). Interestingly, in the only available cell lineage study on crustacean biramous appendages (amphipod pleopods) the two sides, anterior and posterior, of both rami were found to be composed of clonally distinct cell populations (Wolff and Scholtz, 2008). This finding provides a possible developmental basis, among other possible, for a different segmental patterning of the different sides of the same arthropod appendage.

4.4. 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 of the antennal exopod of *Artemia* nauplii: both the setal folds and the ringlet folds provide sites for intermediate muscle insertions. Since setal folds and ringlet folds are not serially arranged in a concordant manner, this is reflected also in the pattern of muscle insertion on the opposite sides (Figs. 4 and 6).

The evolution of the developmental relationship between muscle insertions and appendage joints or trunk segment articulations is far from clear, and specific studies for the appendages are lacking (see Williams and 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 development of the muscles that would attach there (Fournier, 1968). In addition, the molecular mechanism for the development of the joints and muscle insertions is partially similar (Soler et al., 2004). The naupliar antennal exopod of *Artemia* cannot be subdivided into articles (or annuli) since cuticular folds do not produce complete rings, but a close developmental relationship between cuticular folds and muscle insertions seems to be present anyway. However, a close developmental relationship between muscle insertions and joints is far from universal since joints without muscle insertions are rather common in arthropod appendages (reviewed in Boxshall, 2004).

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