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

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

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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 with understanding general properties of the regeneration process, such as the distinction between epimorphosis and morphallaxis and the relationship between normal development and regeneration.

Keywords: Regeneration, antennal flagellum, development, segmentation

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; Brokkes et al. 2001; Galis et al. 2003; Brokkes & 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 (Figure 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. 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.

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 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 to 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 joint 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 a few basic processes: apicalization, resorption, incomplete division of a quartet, and division of the (new) terminal article.

Apicalization. This process occurs always, irrespective of the amputation level in the flagellum. Apicalization is the transformation into a new apex of the distalmost article left after the amputation (Figure 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 (Figure 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% of 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 contralateral, 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 (Figure 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 Figure 4). This outcome was observed rarely (five
cases), and was obviously limited to amputations in the meristematic region.

**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 figure 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.

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

**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 (data not shown).

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.
Occurrence of apicalization and resorption of articles

While apicalization always occurs, 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 do 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 joint are not produced again.

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 also presented other

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<th>Developmental context</th>
<th>Composition of the apical complex</th>
<th>Number of observed cases</th>
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<td>Normal development</td>
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<td>see Maruzzo et al. (2007)</td>
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<td>Regeneration from PBP</td>
<td>q&lt;sub&gt;1&lt;/sub&gt; q&lt;sub&gt;2&lt;/sub&gt; q&lt;sub&gt;1/3&lt;/sub&gt; Apex</td>
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<td>Regeneration following flagellar amputation (N=64)</td>
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[Table I. Summary of the apical complexes emerging from normal development (undamaged antennae), regeneration from the preferred breakage point (PBP), and regeneration following flagellar amputation. q<sub>i</sub>, q<sub>j</sub>, q<sub>k</sub>, and q<sub>4</sub> 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<sub>1</sub> and q<sub>3</sub> 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<sub>1/3</sub> indicates an article that according to the setal pattern could be identified as either q<sub>1</sub> or q<sub>3</sub>. In subapical position, a q<sub>1/3</sub> can be either produced by division of the new terminal article or be already present (as q<sub>1</sub> or q<sub>3</sub>) but becoming subapical only after apicalization of the next article distal to it. Formulas in square brackets specify the origin of these articles: q<sub>subA</sub> is an article with setal pattern q<sup>1,4</sup>, but produced by the division of the new terminal article; x<sub>subA</sub> is an article with abnormal setal pattern produced by the division of the new terminal article; q<sup>1,4</sup> is the distal member of a two-article incomplete quartet; q<sup>1,2,3,4</sup> is a one-article incomplete quartet, i.e., a potential quartet founder, as released from the meristematic region (both q<sup>1,4</sup> and q<sup>1,2,3,4</sup> exhibit the setal pattern of q<sub>4</sub>; see Figure 4). 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 Figure 5). 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.]

Discussion

Occurrence of apicalization and resorption of articles

Antennal regeneration in *A. aquaticus*
uneven 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 Figure 5.

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 Schäfer (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 do not seem to change the morphogenetic activity of the meristematic zone (Schäfer 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 contralateral 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 to 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 conditions. 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 are 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 mitoses are probably localized in the basalmost part of the flagellum. If so, the mechanism by 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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