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MORPHOGENESIS

Morphogenesis in Colonial Hydroids: Pulsating Rudiment Splitting

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Abstract—Two spatially separated processes underlie the growth and morphogenesis in hydroids (Cnidaria, Hydroidomedusa): (1) growth pulsations of the terminal growing tips and (2) cell proliferation and migration in more proximal parts of the colony soft tissues. Growing tips are morphogenetic elements of the colony that provide for the colony elongation and morphogenesis. In thecate hydroids (subclass Leptomedusae) with highly integrated colonies and monopodial shoot growth, the initiation of the lateral branches and hydranth rudiments looks like a periodic splitting of the growing tip into two or more rudiments. Published descriptions and proposed models of this process assume that the splitting results from the formation of the furrows running into the tip from its apical surface. In this study on a Sertulariidae species, we demonstrate that the visible process of the tip splitting into several rudiments begins in its proximal part. At the same time, the inner ridges are initiated at the skeleton lateral surfaces surrounding the growing tip. These ridges develop and grow along the proximodistal axis. Eventually, the opposite ridges fuse, which splits the tip into several rudiments. We propose that the tip splitting into several rudiments is impossible without the spatial regulation of the outer skeleton formation. This process explains many species-specific properties of the shoot spatial organization in thecate hydroids such as the partial or complete fusion of the zooid skeleton with the shoot stem skeleton, deflection of the distal parts of the zooid skeleton from the shoot stem axis, etc. The revealed mechanisms considerably supplements and corrects the models describing morphogenesis in colonial hydroids.

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Similar to plants, colonial hydroids (Cnidaria, Hydroidomedusa) have modular organization (Rosen, 1979) and are one of the simplest multicellular animals. Cyclic morphogenesis repeated many times during the colony life makes colonial hydroids a convenient model to study the rules and mechanisms of morphogenesis. The soft body in hydroids includes only two cell layers, epidermis and gastrodermis. The colony body can be presented as a branching two-layer tube. A part of the colony branching tubular body, hydrorhizal stolons, is used to attach to substrate. The upper sides of stolons contain either feeding zooids (hydranths) or shoots at a certain distance from each other; the shoots in turn contain numerous hydrants connected by the colony tubular body. The termini of branches (stolons or shoots) are occupied either by feeding zooids (hydranths) or by morphogenetic elements of the colony—growing tips (Crowell and Wyttenbach, 1957; Davis, 1971; Kosevich, 1990). From the outside, the surface of the colony soft body (coenosarc) is covered with the external rigid chitinious skeleton (perisarc) (Naumov, 1960).

The growing tips provide for the morphogenesis and elongation of the colony branches, while the direct body growth is mediated by cell proliferation in the coenosarc proximal to the growing tips (Wyttenbach, 1965; Kosevich, 1999). Thus, growth and morphogenesis are spatially separated in colonial hydroids (Belousov, 1961).

The morphogenetic movements of colonial hydroids rely on growth pulsations of the growing tips—periodic extensions and retractions of the tip apex driven by the reorientation of epidermal and gastrodermal cells in the proper growing tip (Zaraiskii et al., 1984; Labas et al., 1992; Beloussov et al., 1993; Kosevich, 2006). The outer skeleton, perisarc, plays an important role in growth pulsations and morphogenesis of colonial hydroids of the subclass Leptomedusae. The new perisarc is excreted at the very apex of the growing tip and hardens on its sides. After hardening, the perisarc shape cannot be changed; that is why the regulation of the relative rates of apical growth and hardening of newly laid perisarc are crucial for the morphogenesis in thecate hydroids (Kossevitch et al., 2001; Berking et al., 2002; Kosevich, 2006).

In all cases, the formation of a new colony element starts from the initiation of a new growing tip. In the case of shoots, the growing tip emergence is followed by a cycle of morphogenetic processes giving rise to a new internode carrying one or several hydranths. Many colonial hydroid species (Sertulariidae, Plumulariidae,

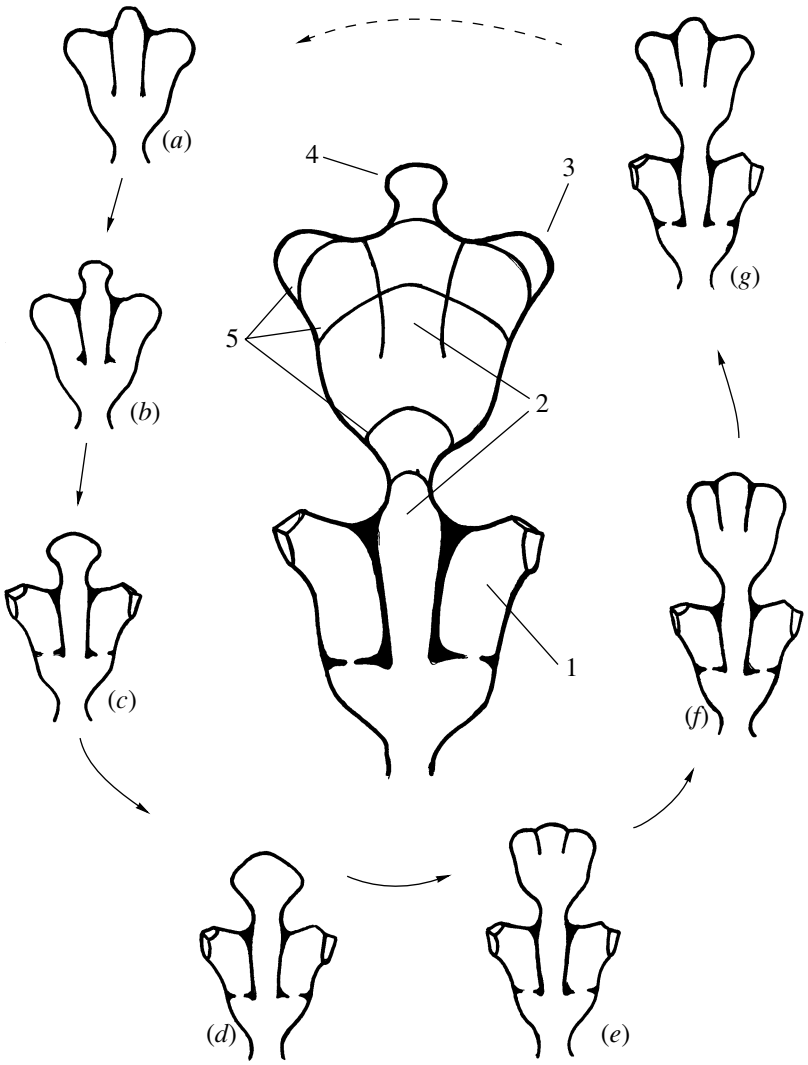

Fig. 1. Successive stages (*a*)–(*g*) of periodic shoot internode development in *Dynamena pumila* (after Marfenin, 1993). Center, schematic structure of the distal part of the shoot with two internodes (only the outer skeleton is shown): *1*, normal hydrotheca of formed hydranth; *2*, shoot stem (axis); *3*, forming hydranth; *4*, growing tip during early morphogenesis; *5*, successive outlines of typical developing internode.

Aglaopheniidae, etc.) demonstrate the "monopodial shoot growth" (Kuhn, 1914; Naumov, 1960). Such shoots have a long-term growing tip that is regularly split into several rudiments, one of which remains a growing tip and continues to form the shoot axis, while other one(s) differentiate into hydranths or growing tips of the shoot lateral branches (Berrill, 1949; Berking et al., 2002; Marfenin and Kosevich, 2004).

The cyclic process of internode formation in a shoot with monopodial growth has been studied best in thecate colonial hydroid *Dynamena pumila* L. (Sertulariidae, Leptomedusae) (Berrill, 1949; Belousov, 1965, 1975; Belousov and Dorfman, 1974; Marfenin, 1975; Berking et al., 2002). During the shoot internode formation in *D. pumila*, the initially hemispherical growing tip expands in the shoot plane and splits into three rudiments (Fig. 1). The central rudiment continues to function as a shoot growing tip, while the lateral ones give rise to a pair of opposite hydrants. The detailed analysis of shoot internode development in *D. pumila* was used to model morphogenesis in colonial hydroids (Belousov, 1965, 1968, 1975; Beloussov, 1991; Beloussov and Grabovsky, 2003) and to simulate the regulation of the structural pattern in hydroids (Berking et al., 2002; Berking, 2006).

In nearly all studies, the process of tip splitting into three rudiments was attributed to the formation of vertical furrows on the apical surface of the growing tip (Belousov, 1965; Beloussov, 1973). It looked like the division of the apical surface into three by furrows running into it to form three neighboring and interacting rudiments. Hence, it was assumed but not considered in detail that, during furrow formation and rudiment growth, the perisarc walls are formed, which fix the tip

splitting (Figs. 1e–1g). Stated differently, most publications assumed that the visible splitting of the growing tip tissues in *D. pumila* is largely initiated in the apical surface, and three daughter rudiments develop independently although in close interaction (Belousov, 1975).

In our view, such scenario does not completely agree with the recently revealed features of colonial hydroid growth and complicates the explanation of some morphological features of their shoots. Accordingly, we tried to analyze in detail the changes in the apical structure of the growing tip in *D. pumila* throughout the internode development. The data obtained suggest that the splitting of the growing tip tissues in *D. pumila* into three rudiments starts from the lateral surfaces rather than from the apical surface. The major mechanism underlying this splitting is the regulation of the spatially uneven excretion and hardening of the perisarc around the growing tip periphery. A scenario of the growing tip splitting into several rudiments is proposed, which explains a number of typical morphological features of shoots in thecate colonial hydroids.

MATERIALS AND METHODS

Subject of study. Experiments were carried out on colonial hydroid *Dynamena pumila* L. (Sertulariidae). This representative of thecate hydroids (Leptomedusa, Hydroidomedusa) (Bouillon and Boero, 2000) features monopodial growth of the shoots with terminal growth zones (Fig. 2a). Similar to most Sertulariidae, the hydrothecae in *D. pumila* have no pedicel and partially fuse with the perisarc of the shoot stem. They are bilaterally symmetrical and have an aperture closed by an opercular apparatus (Fig. 2b). A shoot stem section with two opposite hydrothecae partially fused with the stem compose a shoot internode. Neighboring internodes are separated by narrow shoot stem sections without hydrothecae and often with a circular constriction on the perisarc. The internode length is 0.3–0.8 mm. The shoot internodes are flattened and their planes coincide (Fig. 2c). Internode cross sections at the hydrotheca level demonstrate that the hydrothecae are not strictly opposite relative to the shoot stem: the vertical symmetry planes of opposite hydrothecae have an angle of less than 180°. They are slightly shifted in the direction of stolon growth; hence, the shoot internodes and the whole shoot are bilaterally symmetrical in the strict sense (Fig. 2d). For simplicity, let us assume that the hydrothecae are strictly opposite and the internode planes form the frontal plane of the shoot that is perpendicular to the real symmetry plane of the shoot (discussion of the factors underlying the internode bilateral symmetry in *D. pumila* goes beyond the frames of this study).

Histological processing. Shoots with growing tips at different stages of internode formation were isolated from colonies collected in natural habitats. The material was fixed with 2.5% glutaraldehyde in phosphate buffer, pH 7.4 (Millonig, 1964) at 4°C for 1 h, postfixed with 1% OsO_4 in the same buffer, and stored in fresh glutaraldehyde in the same buffer at 4°C until embedding in resin.

Shoot fragments with one or two distal internodes were dehydrated in graded series of ethanol and acetone and embedded in a mixture of Epon (Fluka, Switzerland) and Araldite (Serva, Germany). Series of semithin sections (1–2 μm) were cut on ultratomes MT 5000 (Dupont Sorvall, United States) and UMTP-3 (Soviet Union). The sections were stained with a mixture of toluidine blue and methylene blue (Mironov et al., 1994).

The sections were examined and photographed using an Axioplan 2 Imaging microscope (Zeiss, Germany) equipped with an AxioCam HRm camera (Zeiss, Germany). Images were processed using the AxioVision and Adobe Photoshop software.

Registration of growth pulsations. The whole cycle of the shoot internode formation in *D. pumila* at 16–18°C is completed in 36–72 h. Therefore, growth pulsations were registered during the shoot internode formation in several stages using several shoots isolated from colonies maintained under laboratory conditions. The shoots in a 50–100 ml chamber were placed under a Biolam 2 microscope (50×–200×) equipped with a WV-CP610/G video-camera (Panasonic, Japan) attached to an AG-6040 time-lapse video recorder (Panasonic, Japan). The period of growth pulsations and the movement direction of the apexes in the rudiments were analyzed in the records.

RESULTS

Morphological changes in the growing tip during the shoot internode formation. For convenience, we recognized five stages in the morphogenetic cycle with more or less clear limits (Fig. 3). *The first stage* of the morphogenetic cycle starts with the emergence of the next internode rudiment (growing tip) on the top of the central part of the last formed internode. By the end of the first stage, it becomes nearly spherical and completely corresponds to the apical part of the growing tip (Fig. 3a).

The changes in the growing rudiment symmetry correspond to the transition from the first morphogenetic stage to the second one. During the *second stage,* the rudiment increases in size and acquires the shape of a flattened inverse cone. The rudiment is flattened through the growth in the planes coinciding with the frontal planes of symmetry of hydrothecae in previous internodes (conventionally, in the shoot frontal plane). At the same time, the rudiment with initially radial symmetry retains a single symmetry plane (Fig. 3b). During the second stage, the common gastral cavity of the shoot grows into the rudiment as its size increases, and now the rudiment corresponds to a distal part of the growing tip. The perisarc starts to thicken in the lower

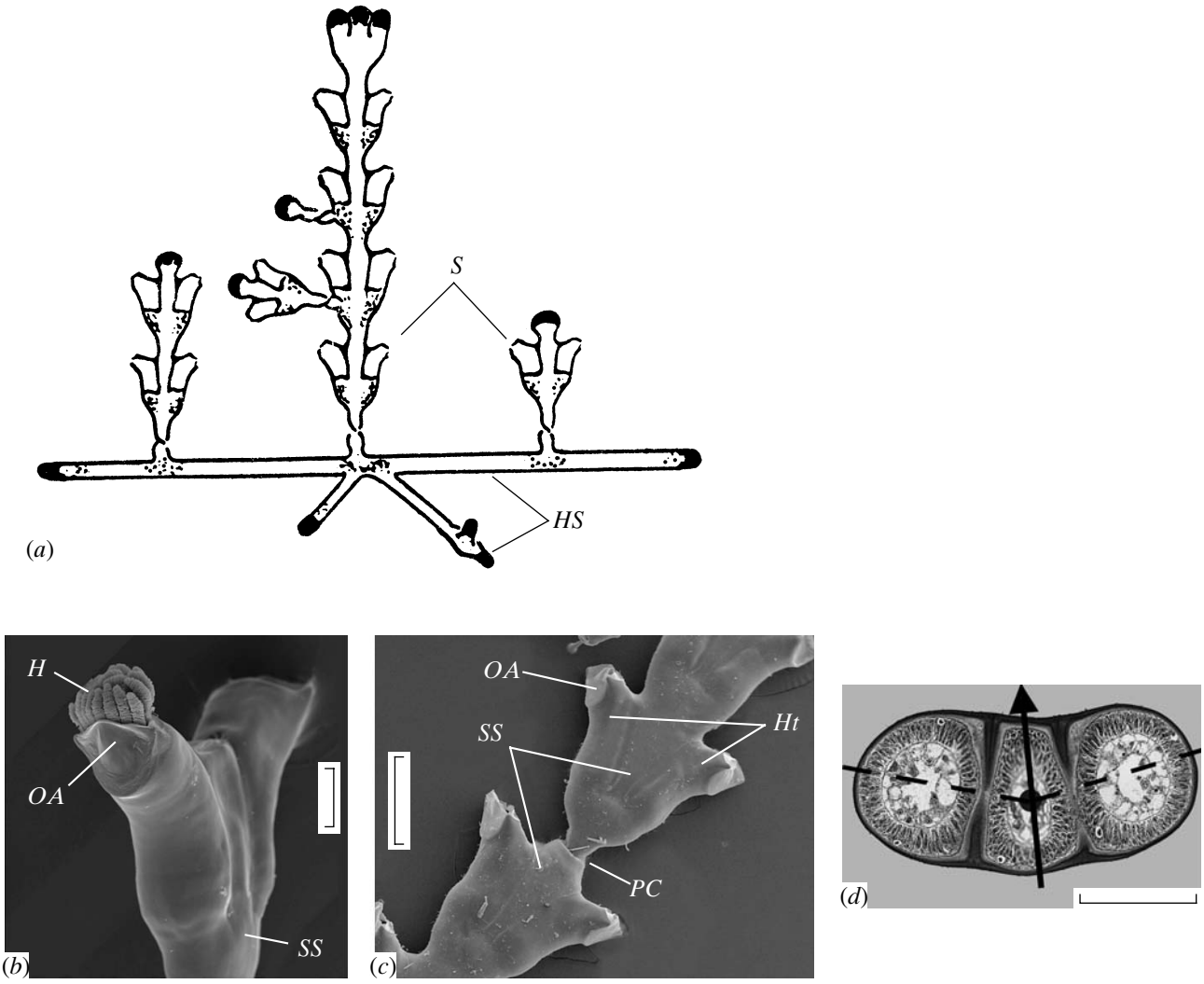

Fig. 2. Structure of *Dynamena pumila* colony: (*a*), monopodial shoot growth (schematic);(*b),* shoot internode (side view); (*c*), shoot fragment with two formed internodes; (*d*), internode symmetry planes. *H, hydranth*; *Ht*, hydrotheca; *OA*, opercular apparatus; *PC*, perisarc constriction between successive internodes; *S*, shoot; *HS*, hydrorhizal stolon; *SS*, shoot stem; ■, zones of growth and morphogenesis; ⁝⁝⁝, branching zones (*a*); (⟶, vertical symmetry plane of the shoot showing the growth direction of the stolon with the shoot; - - - -, vertical symmetry planes of the internode hydrothecae. Scale: (*b*) and (*d*), 100 μm; c, 300 μm.

parts of the growing internode, particularly, on the lateral surfaces.

The transition to *the third stage* of internode morphogenesis features the visible appearance of two perisarc walls in the distal part of the rudiment, which divides it into three parts. When observed in the frontal plane in transmitted light, the walls look oriented in the proximodistal direction (Fig. 3c). As the internode is formed, the walls elongate distalward, thus, separating the rudiment more and more. By the end of the third stage, the tip is completely divided into three rudiments: two lateral ones (that give rise to hydranths later) and the central one (that continues the shoot growth) (Fig. 3d). During almost the whole third stage, the apical part of the growing tip continues to pulsate as a whole.

Analysis of the internode structure at the end of the third morphogenetic stage demonstrates that the walls dividing its tip are bent symmetrically to each other relative to the shoot symmetry plane. The proximal parts of the perisarc walls formed in the early third stage are oriented at an acute angle to each other. As internode grows, the relative orientation of the walls approaches a parallel orientation, and later their distal ends start to diverge in opposite directions (Fig. 4).

During the preceding morphogenetic stages, the developing internode had a structure typical of the growing tip (Hale, 1960; Kosevich, 1991, 2006): its tissues remained in contact with the perisarc throughout the entire surface of the developing internode. At the end of the third morphogenetic stage, the tissues in the proximal part of the developing internode lose contact

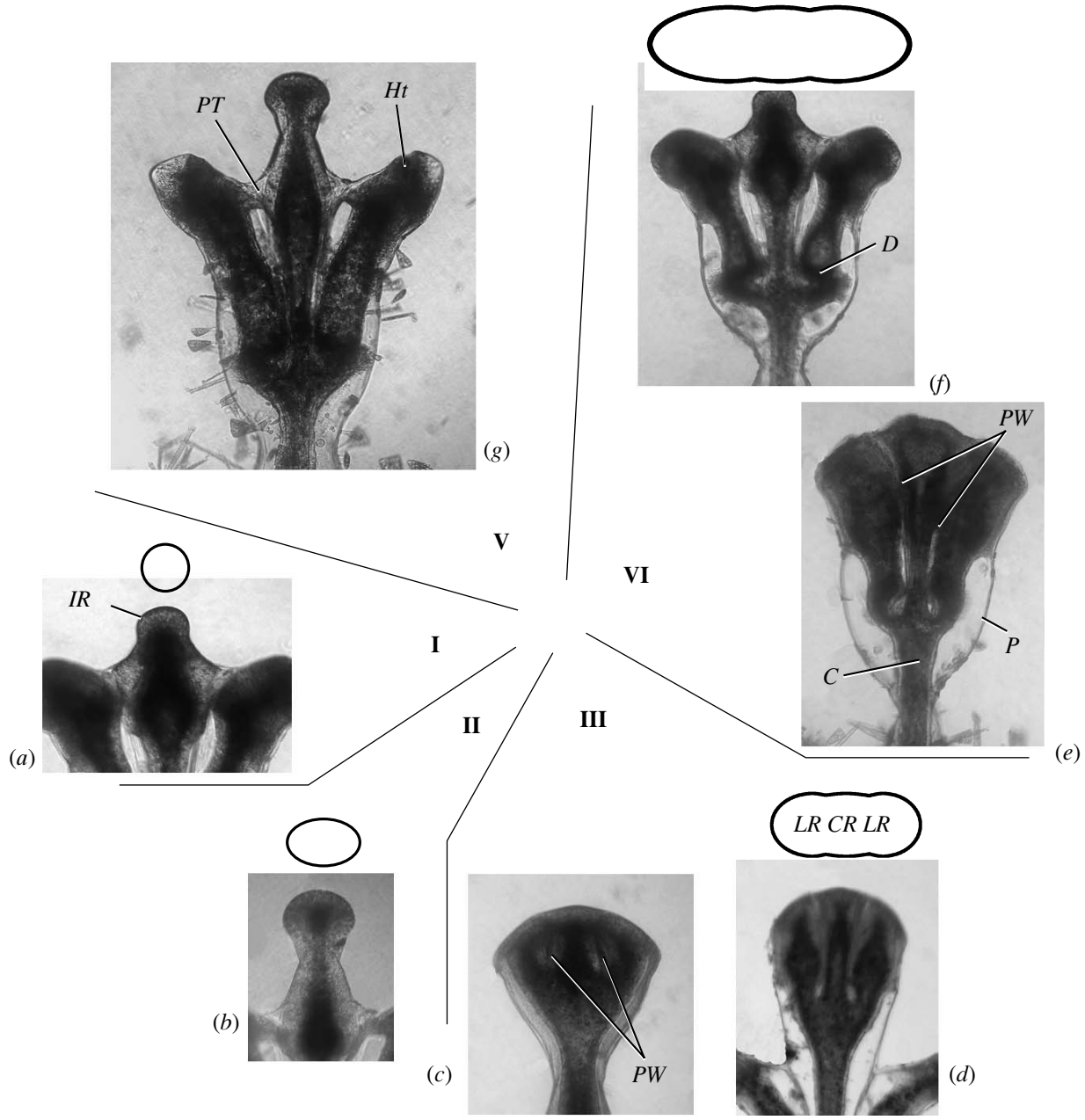

Fig. 3. Morphogenetic cycle of shoot internode in *Dynamena pumila.* Successive changes in the rudiment external morphology (side view) (*a*)–(*g*); (*a),* (*b*), (*d*), and (*f*), rudiment outline, top view; **I–V** main stages of the cycle. *LR*, lateral rudiment; D, diaphragm; *IR*, internode rudiment; *P*, perisarc; *PW*, perisarc wall; *PT,* perisarc thickening; *C*, coenosarc; *CR*, central rudiment; for other designations, see Fig. 2.

with the surrounding perisarc (Figs. 3d and 4c). Hence, only the distal part of the internode rudiment corresponds to the growing tip by this period.

The onset of *the fourth stage* corresponds to the asynchronization of the apical surface pulsations of the lateral and central rudiments. After their complete splitting, three clearly separate protrusions emerge on the apical surface of the tip, which correspond to the distal parts of the growing rudiments. The concave regions on the apical surface of the tip corresponds to the sites where the walls that separate the rudiments merge with the perisarc of the former apical surface of the growing tip (Fig. 3e).

Soon after the fourth stage starts, the tissues adjacent to the perisarc walls in the central part of the rudiments lose contact with them. In the distal parts of all three rudiments, the coenosarc remains in tight contact with the proximalmost regions of the perisarc walls (Fig. 3f). At the same time, the outer diameter and the lumen of the cylinder of tissues lying free in the lateral

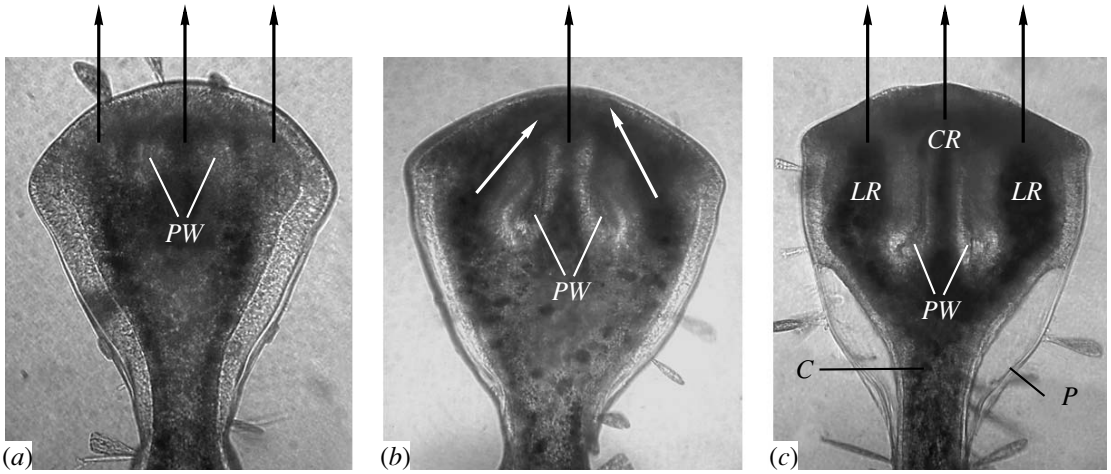

Fig. 4. Changes in shape of perisarc walls and pulsation directions of primordia during third stage of morphogenetic cycle of shoot internode in *Dynamena pumila:* (*a*), beginning (emergence of perisarc walls); (*b*), middle; (*c*), end; ⟶, direction of rudiment pulsations; for other designations, see Fig. 3.

rudiments are substantially reduced. The process of lateral rudiment differentiation into hydranth starts. The diameter of tissues in the central rudiment also substantially reduces. At the end of the fourth stage, the distal parts of the lateral rudiments face away from the direction of the shoot axis growth. The developing internode reaches the final size, and the elongation of the lateral rudiments stops. This state corresponds to the transition to *the fifth stage* of internode morphogenesis.

This stage mainly includes the processes of lateral rudiment differentiation into hydranth (Fig. 3g). The diaphragm starts formation from the proximal end of the inner longitudinal wall and expands towards the outer perisarc of the lateral rudiment. In the vicinity of the internode lateral wall, the diaphragm has a small opening, through which the coenosarcal canal connect the hydranth with the coenosarc of the shoot axis. In the distal part, the circular region of continuous contact with the perisarc is maintained in the developing hydranth for a long period. At the same time, the upper part of the adjacent hydrotheca side (i.e., the region of hydrotheca deflection from the shoot axis) has a clear and significant perisarc thickening (Fig. 3g). Above it, there is a free hydrotheca part, on the distal surface of which the hydranth opercular apparatus is formed. At the final stage of differentiation, the tentacles and hypostome of the hydranths are formed. The completely formed hydranth breaks the thin perisarc connecting the opercular flaps, after which it can protrude from the hydrotheca.

The structure of the coenosarc in the central part of the internode remains unaltered. Under favorable conditions, the next growing tip of the shoot internode is formed in the distal part at the end of the fourth stage. Such morphological changes are observed during the morphogenetic cycle of each internode in the *D. pumila* shoot.

Changes in the pattern of growth pulsations during the morphogenetic cycle. The pattern of the rudiment pulsations consecutively changes in the course of the morphogenetic cycle. After the initial stages, the rudiment demonstrates the typical growth pulsations pattern with a long extension phase, when the rudiment length remains constant (Wyttenbach et al., 1973; Belousov et al., 1984; Beloussov, 1991). The mean pulsation period is 11–13 min at 16–18°C. The video recording data demonstrates that the growth pulsations pattern does not significantly differ after the perisarc walls emerging at the beginning of the third stage.

Even after the walls separating the rudiments become visible, the internodes pulsate synchronously up to the end of the third stage. Immediately after the walls become visible, the pulsation direction of all three rudiments is the same and is parallel to the longitudinal axis of the shoot. As the internode grows and the walls elongate, the relative pulsation directions of the apexes of the lateral rudiments change (Fig. 4). In the middle of the third stage, pulsations of the lateral rudiments are directed to the shoot axis: the distal parts of the lateral rudiments as though compress the central rudiment from both sides. At the end of this stage, pulsations of the distal parts of the lateral rudiments become parallel to those of the central rudiment again.

At the end of the third stage of internode morphogenesis, the rudiments lose the pulsation synchronism. This is manifested as a delay of the central rudiment in the extension phase relative to the lateral rudiments. During the subsequent development, the rudiments can pulsate asynchronously; however, no strict pattern of their pulsation has been revealed. The pulsation period proved slightly shorter in the lateral rudiments than in the central one; accordingly, the extension and retraction phases of the neighboring rudiments continuously shifted in time relative to each other (Fig. 5). Moreover,

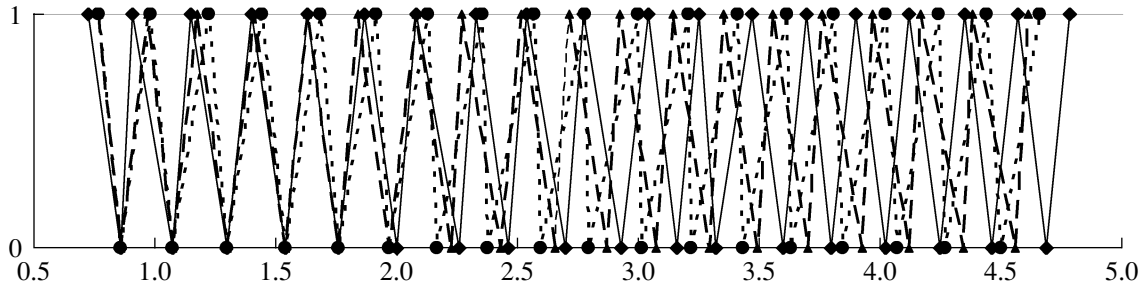

Fig. 5. Proportion between pulsations of the central (–◆–) and lateral (- -●- -, - -▲- -) rudiments of *Dynamena pumila* shoot at the fourth stage. Abscissa: time, h; ordinate: 0, low point (the highest compression of the rudiment apex); 1, peak (the highest extension of the apex).

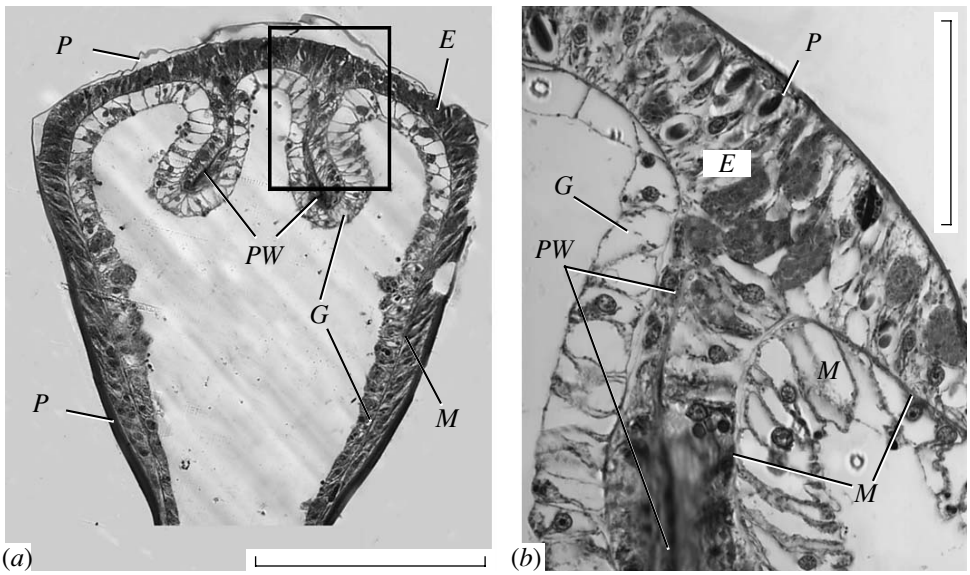

Fig. 6. Longitudinal section along internode axis in *Dynamena pumila* in the middle of the third stage of internode development: (*a*), general view; (*b*), magnified region from (*a*); *M*, mesoglea; *E*, epidermis; *G*, gastrodermis; for other designations, see Fig. 3. Scale: (*a*), 200 μm; (*b*), 50 μm.

even two lateral rudiments can pulsate asynchronously. A detailed analysis of the growing tip pulsation during stages three to five demonstrated a slightly different pulsation period in all rudiments, which also varied in each rudiment. In addition, the rudiments could vary in the proportion between the pulsation phase durations within each cycle.

Histological data. The sequence of the growing tip splitting into three rudiments has been studied on serial sections of the developing internode during different morphogenetic stages.

The perisarc wall emergence initiated proximodistally at a distance from the tip apical surface could be observed on the longitudinal sections starting from the third stage (Fig. 6). A series of longitudinal sections in the middle of the third stage demonstrates the absence of the perisarc walls in the apical part of the tip (Fig. 6b). Two perisarc walls inclined relative to the longitudinal axis of the tip growth are clearly visible in the more proximal region close to the central part of the growing tip. The proximal parts of these walls are thick, while their distal parts gradually become thinner and fade away without reaching the apical surface of the tip.

The perisarc walls are covered with an epidermal loop, which becomes a single whole on the apical surface of the tip. At the site where the epidermal loop covering the walls contacts the epidermis of the tip apex, the epidermis looks multilayered; however, the nucleus is not visible in some cells (Fig. 6b). This can indicate that the observed "multilayered" epidermis actually results from the cell layer bending.

The cross-sections of the tip apex in the middle of the third stage (without the gastric cavity of the developing internode) lack perisarc structures splitting the tip tissues (Figs. 7a–7c). Two pairs of thin perisarc ridges (walls), symmetrical relative to the sagittal symmetry plane of the developing internode, can be found proximally on the opposite frontal walls (Fig. 7d).

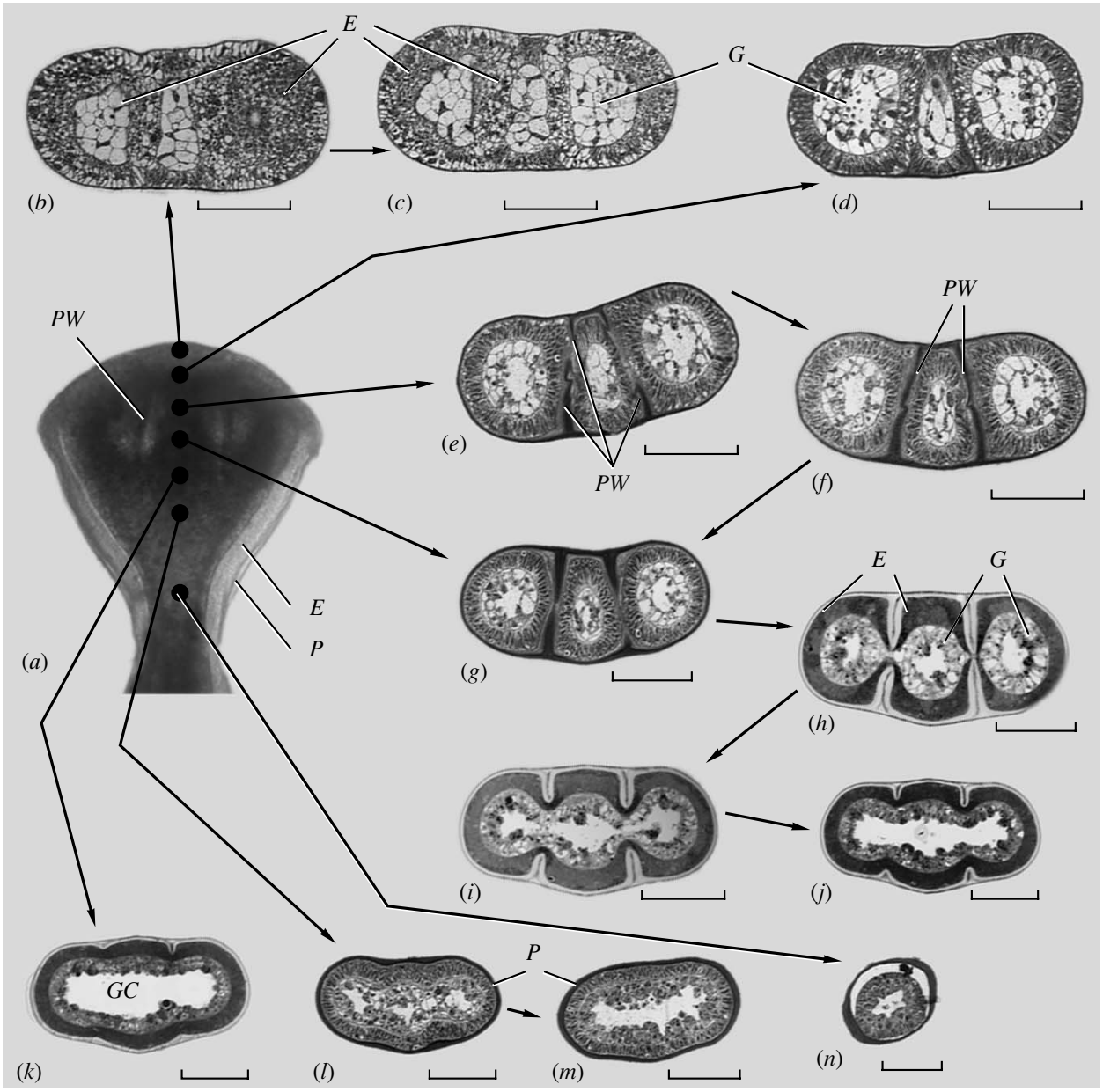

Fig. 7. Cross sections of *Dynamena pumila* internode in the middle of the third stage of its development: (*a*), general side view; (*b*)–(*n*), serial sections from the tip apex to the internode basis (dots mark the levels of the corresponding sections indicated by arrows going from dots; arrows between sections show the sequence of remote sections). *GC*, gastric cavity; for other designations, see Fig. 3. Scale: 100 μm.

Closer to the internode base, they rapidly increase in height and thickness, and each pair of opposite ridges soon merge (Figs. 7e and 7f). In the middle of the third stage, the site of the proximally merging lies in the upper third of the developing internode. No ridge merging is observed, and they gradually disappear (Figs. 7g–7k). It is of interest that the basal part of the wide region in the developing internode with no perisarc ridges has clearly visible division of the perisarc corresponding to the above rudiments (Fig. 7l). The outer longitudinal furrows correspond to slightly thickened perisarc walls (Figs. 2b, 2c, 7k, and 7l).

The soft tissue structure in the growing tip changes as the perisarc ridges develop. When they appear, the cavity of the tip is divided into three longitudinal canals connected by converging lumina. The ridge growth leads to the merging, first, of the gastrodermis of the opposite frontal walls of the tip, and then, of the epidermis (Figs. 7g–7i). As a result, the epidermis and gastro-

dermis of future rudiments are separated at the level of merged regions (formed perisarc walls).

During the third stage, the perisarc walls elongate and gradually reach the apical surface. Since their formation starts from the frontal walls of the perisarc, the epidermis splitting according to future rudiments in the distal part of the tip (near the apex) initially proceeds at the periphery, and it remains undivided in the central part (cf. Figs. 6a and 7b–7d). At the end of the third stage, thin perisarc walls connected with the apical perisarc can be observed on the cross and longitudinal sections of the tip distal part. At the same time, the epidermis of the central and lateral rudiments splits, which is visible as the different staining of the rudiment tissues.

Longitudinal sections of the internode at the fourth and fifth stages demonstrate significant secondary thickening of the perisarc at the proximal ends of the perisarc walls before and during the formation of hydranth diaphragms and at their distal ends in the sites of hydrotheca branching from the shoot axis (Fig. 8). In other regions of the developing internode, the perisarc thickness remains nearly constant. The difference between the primary perisarc laid during its shaping and the secondary perisarc excreted during the subsequent rudiment growth and differentiation is clear even without using special histochemical techniques.

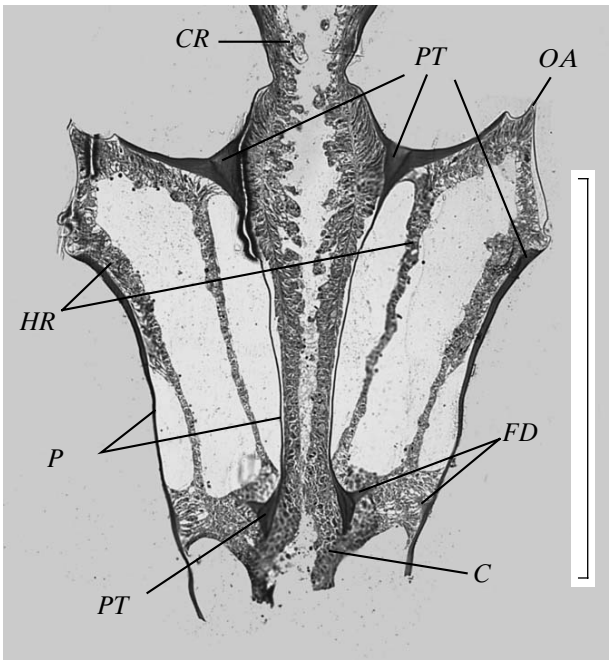

Fig. 8. Longitudinal section along internode axis in *Dynamena pumila* in the beginning of the fifth stage of internode development (the regions of perisarc secondary thickening are clearly visible). *HR*, hydranth rudiment; *FD*, forming diaphragm; for other designations, see Fig. 3. Scale: 500 μm.

DISCUSSION

The internode formation in *D. pumila* and other thecate hydroids (Leptomedusae) with monopodial shoot growth was described many times using visual observation in transmitted light or histological data (Berrill, 1949; Belousov, 1965, 1967; Beloussov et al., 1972; Beloussov, 1973). The interpretation of morphogenesis during the tip growth and the corresponding morphogenetic models in hydroids proposed by Belousov et al. (Belousov, 1967; Beloussov, 1973; Beloussov and Dorfman, 1974; Beloussov, 1991; Beloussov and Grabovsky, 2003) relied on the assumption that the growing tip is a hollow structure with the walls composed of single layer possessing specific physical properties (thickness, elasticity, etc.). The proposed models were based on the assumption that the actual morphogenesis in hydroids is mediated by growth pulsations. At the same time, Belousov et al. concluded that the growing tip can be split into several daughter rudiments through the changes in the curvature of its apical surface in the absence of external (relative to the tip wall) forces. This assumes that different parts of the apical surface of the growing tip start to pulsate with different intensity, i.e., the apical surface (growing tip) is split into several rudiments by the furrows running into it. This explanation of splitting the apical surface of the growing tip as an integrated pulsating layer (or two layers with coinciding activity; Zaraiskii et al., 1984) looks unlikely to us. Moreover, the logic of this model suggests that the daughter rudiments after splitting should develop with no contact with each other and form noncontiguous parts of the outer skeleton. Otherwise, the perisarc walls should be formed in the distoproximal direction after the tip splitting into daughter rudiments.

It is beyond question that the splitting of the tip tissues into several daughter rudiments is initiated by the processes going in the proper tissues on its own. The triggering signal can be the changes in the mechanical strain in different parts of the layer (Belousov and Mittental', 1992; Belousov et al., 2000) or formation of some gradients of morphogenetic factors (Meinhardt and Gierer, 2000). Actually, the initial stages of the growing tip splitting include the thickening of the epidermal layer at the boundaries of future rudiments. Later it gives rise to short-term small furrows in the epidermis along the rudiment boundaries at the stage of the greatest retraction of the growing tip during growth pulsations (Beloussov, 1973). However, the epidermal surface becomes smooth and the furrows disappear in the subsequent tip extension phases. This is due to the nature of growth pulsations and the active role of the tip gastrodermis in pulsations: the final extension of the tip towards the next peak is mediated by the gastrodermis activity pushing the tip epidermis out from the inside (Kosevich, 2006). Under these conditions, the maintenance of the epidermal furrows on the apical surface of the growing tip in the absence of forces external to the tip tissues is hardly possible. Active pulsations of the tip

make improbable the formation of the vertical perisarc walls starting from the actively pulsating apical surface.

The model proposed by Belousov et al. ignore the role of the laying and hardening of the perisarc (outer skeleton) during morphogenesis. At the same time, we have shown previously that the regulation of the perisarc hardening rate is vital to morphogenesis in thecate hydroids (Kossevitch et al., 2001; Kosevich, 2006).

In this work, the sequence of processes going in the *D. pumila* growing tip during its splitting into several rudiments as well as the role of the outer skeleton in these processes have been analyzed. Detailed analysis of the main stages of internode morphogenesis in *D. pumila* indicates that the actual pattern of processes going in the growing tip differs from that described previously based on visual observations.

We believe that the diagram of processes going in the *D. pumila* growing tip during the morphogenetic cycle of the shoot internode is as follows. At the first stage, the growing tip starts to protrude from the distal part of the previously formed shoot internode. First, the rudiment of the next internode becomes spherical, thus, representing just the apical part of the growing tip (Hale, 1960; Kosevich, 1991, 2006); while it starts to expand in the plane of the previous internode after the transition to the second stage of the cycle. Most likely, this is mediated by the delay of the newly laid perisarc hardening on the lateral sides of the growing tip (Figs. 9a and 9b), while its hardening rate on the frontal surfaces of the tip remains constant (Kossevitch et al., 2001; Kosevich, 2006). This allows the tip tissues to expand in the frontal plane much more than in the perpendicular direction. As a result, the internode rudiment acquires the shape of an inverse cone in the frontal projection and oblong-elliptic in the cross-section in the distal part at the end of the second stage (Fig. 9b).

Starting from the third stage, the size of the growing tip in the frontal plane does not significantly differ, which indicates that the rate of perisarc hardening around the tip apex becomes uniform again (Fig. 9c). At the beginning of the third stage, two pairs of perisarc ridges are initiated on the frontal surfaces of the developing internode in its distal part (slightly proximal to the actively pulsating prominent apical surface of the tip) (Fig. 9d). Two pairs of longitudinal depressions corresponding to the boundaries between future hydrothecae and shoot axis become apparent on the outer frontal surfaces according to the inner ridges (Figs. 2b and 7k–7o). Hence, the perisarc fixes even insignificant changes in the outlines of the growing tip tissues. The proper regions are formed through active perisarc excretion by the tip tissues in the regions corresponding to the boundaries between future rudiments derived from the growing tip. Naturally, the inner splitting of the growing tip and the identification of the boundaries between the rudiments go first (see above), after which the splitting becomes visible as the intensified laying and hardening of the perisarc at the sites of short-term furrows in the lateral wall of the tip at the boundaries between the rudiments. This confirms the importance of preliminary layout of the boundaries between the major parts in the developing body (Meinhardt, 1983; Fontana and Buss, 1994; Green, 1999). Thus, the forming perisarc ridges fix the splitting of the soft tissues in the actively pulsating tip into the corresponding rudiments.

The ridges elongate along the internode longitudinal axis and their height increases towards each other in a pair through active perisarc excretion in the formed tissue folds during the internode development. The increase in the ridge height gradually draws the tissues of the opposite (frontal) sides of the tip together, after which the tip gastrodermis merges and splits into three rudiments (outgrowths) (Fig. 7). The gastrodermis splits relatively rapidly after the perisarc ridge initiation, which looks like splitting of the growing tip by the perisarc walls growing into it when the frontal plane of the developing internode is observed in transmitted light (Figs. 3c, 4a, 4b, and 7a).

Significantly, the perisarc ridge formation provides for the layout fixation of the tip splitting into daughter rudiments, thus, increasing the invagination of the frontal walls of the tip coenosarc. The regulation of the rate of perisarc excretion and hardening plays the leading role in this case. The deep furrow formation on the surface of actively pulsating two-layer rudiment is hardly possible without invagination. At the same time, Cherdantsev (2003) proposed that hydroids lack invagination of cell layers as a morphogenetic process during any developmental stages. The active ingrowth of the perisarc ridges is additionally confirmed by the different layer thickness and shape of epidermal cells along the growing ridges. The epidermis is the thinnest on the ridge top, and the shape of curved cells in this region indicates that the epidermis is mechanically forced into the tip cavity (Figs. 7d and 7h).

The rates of perisarc ridge increase in height and proximodistal elongation correlate with the rate of growth tip elongation. As a result, the opposite ridges in a pair first merge in the proximal part of the tip (Fig. 10), while the epidermis remains undivided for a long period in the distal part. This explains some traits observed in the growth of three rudiments during the third stage of internode development.

For instance, when the perisarc ridges emerge at the beginning of the third stage, the pulsation directions of each of visible rudiments are nearly parallel to each other (the direction of the former tip pulsations is retained) (Figs. 4a and 11a). In the middle of the third stage when perisarc walls of the opposite sides merge in the proximal part of the internode and the gastrodermis is split, the pattern of rudiment pulsations changes. During the final pulsation phase, the direction of the apical parts of the lateral rudiments forms an acute angle to the longitudinal axis of the developing internode and the pulsation direction of the central rudiment:

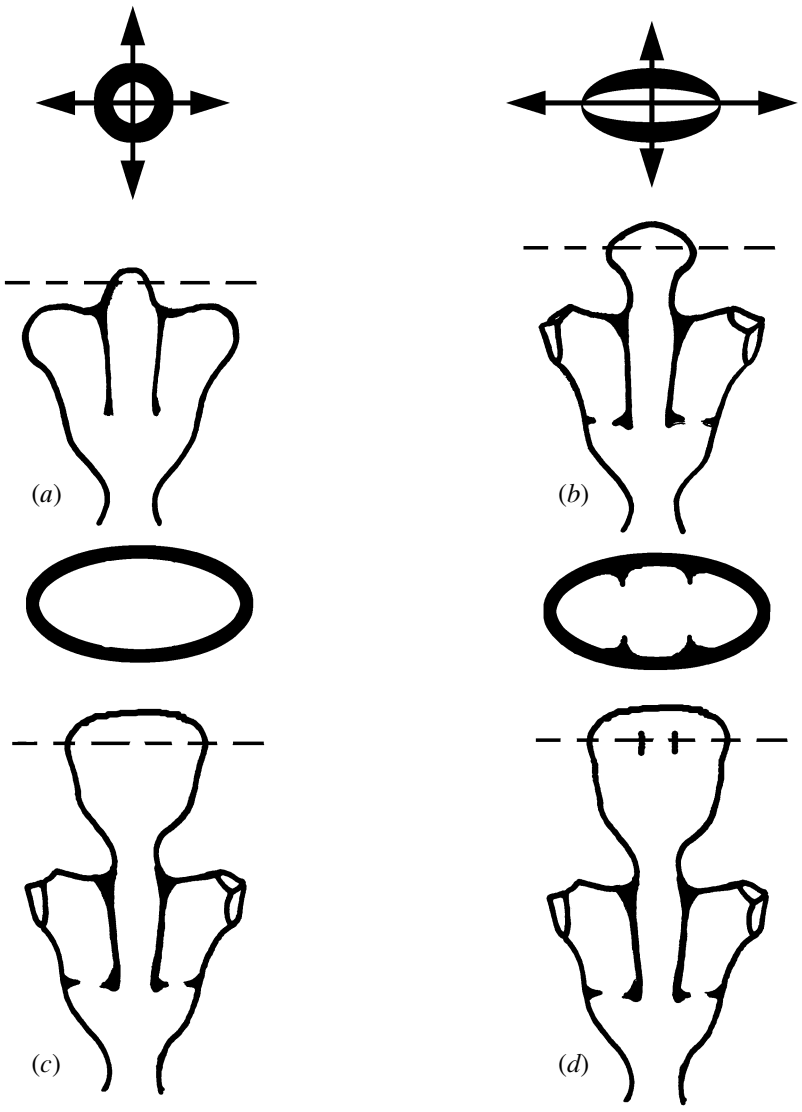

Fig. 9. Schematic changes in shape of cross-section of *Dynamena pumila* internode rudiment during its formation (only perisarc is shown): (*a*), first stage; (*b*), second stage; (*c*), early third stage; (*d*), middle third stage (emergence of inner perisarc ridges). Upper row, cross section (the line thickness corresponds to the rate of perisarc hardening; the arrow length is proportional to the rate of rudiment extension in this direction); lower row, frontal plain view; - - - -, level of the corresponding rudiment cross section.

the lateral rudiments as though compress the central one from both sides (Fig. 4b). This is most likely due to the division of the tip gastrodermis into three rudiments, while the epidermis in the apical part is still an undivided layer. At the final pulsation phase, the distalward extension of the tip apex results from the gastrodermis cylinder elongation (Kosevich, 2006). The capacity of the central rudiment epidermis and, hence, the inner sides of the lateral rudiments to move (extend) distally becomes limited compared to that of the epidermis in the outer sides of the lateral rudiments (Fig. 11b). In addition, the epidermis capacity for distalward movement between the rudiments is limited by its loop covering the already formed perisarc walls in the proximal part of the tip. While the pulsations remain synchronous, this considerably limits epidermis extensibility. As a result, the increasing epidermis extension and displacement on the outer surfaces of the lateral rudiments tilt the movement of the rudiment apexes towards the central rudiment (Figs. 4b and 11b), which fully agrees with the notion of changed growth direction of close rudiments as a result of their interaction (Belousov and Dorfman, 1974).

At the end of the third stage after the perisarc walls are completely formed and the epidermis of three rudiments are separate, the pulsation direction of all three rudiments become nearly parallel to each other and to the longitudinal shoot axis again (Figs. 4c and 11c). By this time, the growing tip corresponds only to the distal part of the forming internode, and its length is shorter than the length of the perisarc walls between the rudi-

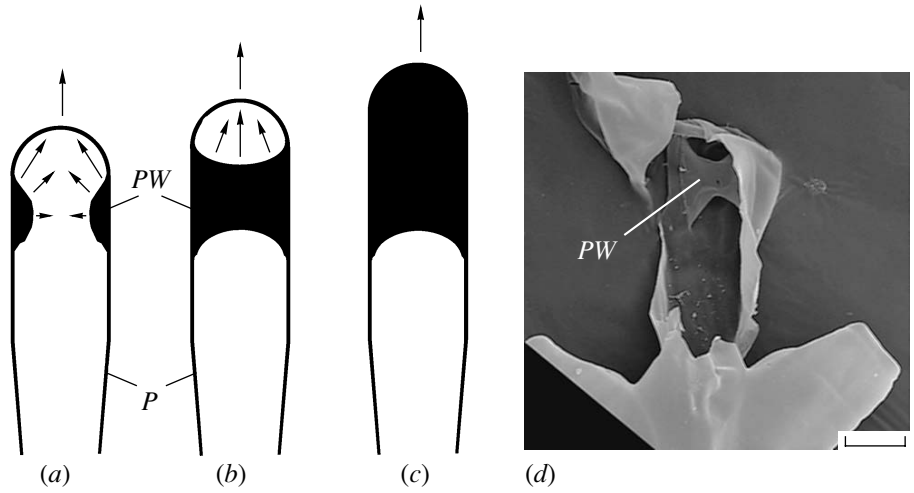

Fig. 10. Schematic development of perisarc ridges in *Dynamena pumila* internode rudiment (cross section of the rudiment along the plane of development of a ridge pair, ■): (*a*), early third stage, emergence of perisarc ridges; *(b)*, middle third stage, ridge fusion; (*c*), late third stage, final ridge formation and complete separation of the rudiments; (*d*), inner part of developing internode in the middle of the third stage (corresponds to (*b*); soft tissues and perisarc of an internode half were removed), scanning electron micrograph. ⟶, direction and rate of tip and ridge growth; the line thickness corresponds to the rate of growth; for other designations, see Fig. 3. Scale: *d*, 100 μm.

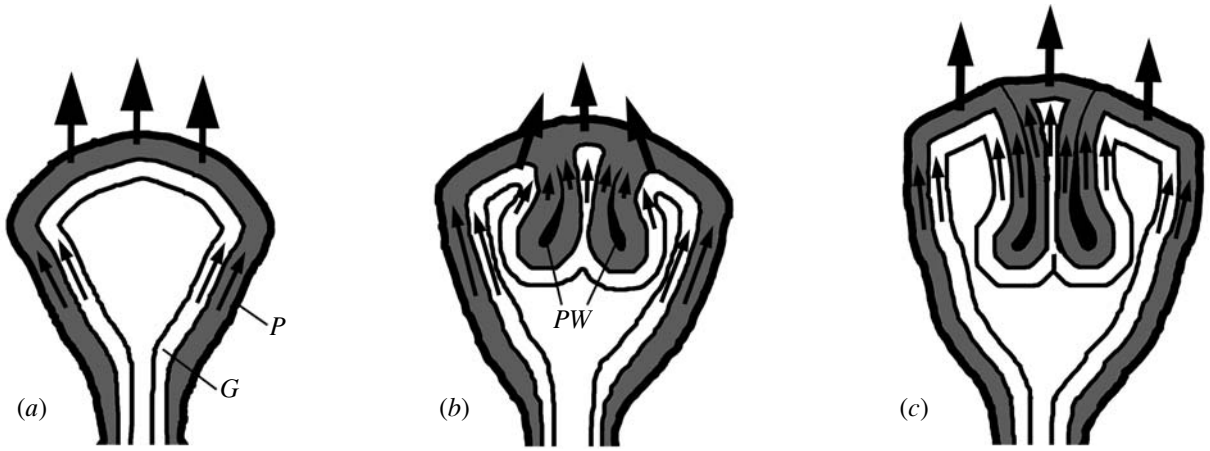

Fig. 11. Schematic changes in direction of layer movements and primordium pulsations during internode rudiment development in *Dynamena pumila* in the beginning (*a*), middle (*b*); fusion of perisarc ridges in the proximal part of the internode), and end of the third stage (*c*); complete separation of the rudiments). (■), epidermis; ⟶, direction of rudiment pulsations; ⟶, direction of say layer movements during growth pulsations; the line length corresponds to the displacement length; four other designations, see Fig. 3.

ments. Under these conditions, the length of the tissues along the formed perisarc walls does not substantially limit the epidermis extension in the pulsating rudiment.

Even after the walls between the rudiments become apparent, the rudiments continue synchronous pulsations up to the end of the third stage. In all rudiments during the active pulsation phase, the extension to the level of the next peak, the tissue forward movements start simultaneously. Later, the central rudiment can be slightly delayed from the lateral ones (that compress the central rudiment, thus, preventing it from reaching the peak simultaneously with the lateral rudiments); however, the pulsation period remains the same in all rudiments. We believe that this indicates that the epidermis in the distal part of the shoot tip remains undivided, i.e., the coenosarc in the apical surface of the growing tip remains undivided up to the end of the third stage.

The onset of the fourth stage commonly coincides with the onset of asynchronous pulsations of all three rudiments. Most likely, this is due to the complete separation of the three rudiments from each other in the distal part of the developing internode and to the splitting of the growing tip into three independent ones.

Owing to their common origin, the new growing tips maintain similar periods of growth pulsations; however, even minor differences eventually lead to notably asynchronous pulsations of rudiments in the developing internode.

During the fourth stage of internode development, the elongating distal parts of the lateral rudiments start to bend away from the central rudiment (Fig. 3e). In our view, the change in the direction of rudiment growth in this case only marginally depends on their interaction involving the cell layer elasticity (Belousov, 1965). We believe that the main factor forcing the lateral rudiments to deflect from the central one in this case is active excretion and accelerated hardening of the perisarc near the distal ends of the perisarc walls between the separated tips. As a result, the apex diameter in the central rudiment slightly decreases (a visible constriction is formed between the shoot internodes), while the apexes of the lateral rudiments bend towards the thinner and softer perisarc. At the final stages of internode development, the difference in the excretion rate and secondary thickening of the perisarc in these sites is clearly visible on longitudinal sections (Fig. 8).

The tip size of the lateral rudiments gradually decreases relative to that of the central rudiment during this stage. At the end of the fourth stage, epidermal cells remain in continuous contact with the perisarc only in the distalmost part of the lateral rudiments and near the proximal ends of the perisarc walls. The continuous contact between the epidermis and perisarc favors its active secondary thickening. As a result, the perisarc walls splitting the central and lateral rudiments appear much thinner in their middle relative to the proximal and distal parts (Fig. 8).

During the fifth stage of internode development, the lateral rudiments growth is arrested and the hydranth differentiation starts. The hydranth diaphragms are formed in the proximal part of the lateral rudiments as additional walls growing from the thickening proximal parts of the longitudinal walls towards the lateral surfaces of the internode perisarc (Figs. 3f, 3g, and 8) due to the local excretion of the perisarc. Tentacles and hypostome of hydranths are differentiated and the hydrotheca opercular apparatus is formed in the distal parts of the lateral rudiments. It is of interest that the region of opercular flaps is not subject to secondary thickening (Fig. 8) even if epidermal cells in this region remain in contact with the perisarc for a long period. This can be considered as an additional indication of spatial regulation of the perisarc secondary thickening.

During the fifth stage, the central rudiment either starts a new cycle of shoot internode development or arrests the development at the stage of vague hemisphere. In the case of the growth arrest, the central rudiment is always observed in this state, and no intermediate variants are normally observed. This indicates that the cycle of shoot internode development in *D. pumila* starts and ends at the stage of vague protrusion on the apical end of the shoot axis between the distal pair of hydrothecae.

The described sequence of events going in the growing tip of *D. pumila* splitting into three rudiments explains the specific internode structure in this species. In addition, the described splitting mechanism of the pulsating rudiment (growing tip) into several rudiments developing in parallel provides the basis to explain the morphological variation in thecate hydroids. The degree of hydrotheca merging to the stem or their "sinking" (Naumov, 1960) into the shoot stem, the deflection of the hydrotheca aperture from the stem axis, and mutual arrangement of hydrotheca and lateral branches of the shoots can be explained in terms of varying several variables crucial for morphogenesis. These variables include (1) relative time of the boundaries and rudiment fate determination, (2) the rate of perisarc excretion and hardening relative to the tip growth rate, and (3) the time of the transition of hydranth rudiments to final differentiation relative to the time of complete rudiment separation from the growing tip of the shoot stem axis. In addition, the spatial regulation of the perisarc excretion and hardening is one of key mechanisms underlying morphogenesis in thecate hydroids. These main parameters allow us to explain most morphological types of the shoot known to date at least in the Sertulariidae family represented by *D. pumila.*

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