

Shaping of Colony Elements in *Laomedea flexuosa* Hinks (Hydrozoa, Thecaphora) Includes a Temporal and Spatial Control of Skeleton Hardening

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Abstract. The colonies of thecate hydroids are covered with a chitinous tubelike outer skeleton, the perisarc. The perisarc shows a species-specific pattern of annuli, curvatures, and smooth parts. This pattern is exclusively formed at the growing tips at which the soft perisarc material is expelled by the underlying epithelium. Just behind the apex of the tip, this material hardens. We treated growing cultures of *Laomedea flexuosa* with substances we suspected would interfere with the hardening of the perisarc (L-cysteine, phenylthiourea) and those we expected would stimulate it (dopamine, *N*-acetyldopamine). We found that the former caused a widening of and the latter a reduction in the diameter of the perisarc tube. At the same time, the length of the structure elements changed so that the volume remained almost constant. We propose that normal development involves a spatial and temporal regulation of the hardening process. When the hardening occurs close to the apex, the diameter of the tube decreases. When it takes place farther from the apex, the innate tendency of the tip tissue to expand causes a widening of the skeleton tube. An oscillation of the position at which hardening takes place causes the formation of annuli.

Introduction

The fragile, almost beautiful pattern of hydrozoa colonies attracts every observer's interest. There are many variations reminiscent of plumes, plants, or minute trees. At closer examination the pattern of the colonies is fixed by a rigid outer skeleton, the chitinous perisarc. We are interested in how this perisarc is shaped.

Inside the perisarc is a hollow tube of soft tissue composed of two cell layers separated by an extracellular matrix, the mesogloea. This matrix is flexible. In general, a colony comprises two parts: a net of tubes (stolons or hydrorhiza) generally fixed to a substratum, and shoots (hydrocauli) emerging vertically from these stolons in a more or less regular pattern. The shoots bear polyps (hydranths) with which the animals catch their prey. All parts of the colony are covered with the perisarc. In thecates, the polyp expands out of the tubelike endings of the perisarc covering (Fig. 1).

The perisarc of the stolons is an almost uniform tube that is flattened at the site of tight contact to the substratum. The perisarc of the shoots in *Laomedea flexuosa* Hinks, used in this research, forms a repetitive pattern (Fig. 1). One element of the shoot—the internode—consists of two sequences of annuli separated by a smooth, slightly bent tube and followed by the finely structured housing (hydrotheca) of the polyp. The sequence, the number, and the size of the pattern elements are almost invariant and species specific (Kosevich, 1990). The exact composition of the perisarc is unknown, but it appears to contain up to 30% of chitin (Jeuniaux, 1963; Holl *et al.*, 1992).

Both the stolon and the shoot tubes increase in length exclusively at their tips (Kühn, 1914; Hyman, 1940). Hence, the pattern of the perisarc emerges exclusively at that site. Close to the apex of the elongating tube, this material is rather soft and flexible. Its shape is exactly that of the underlying tissue. The perisarc material “hardens” some dozens of micrometers proximal to the apex, and from that time onward it has a fixed shape. The pattern of the perisarc is a time recording of the activity of the tissue in the tip.

Our interest is to learn how the perisarc is shaped. One possibility is that in the course of growth, cell-cell interactions

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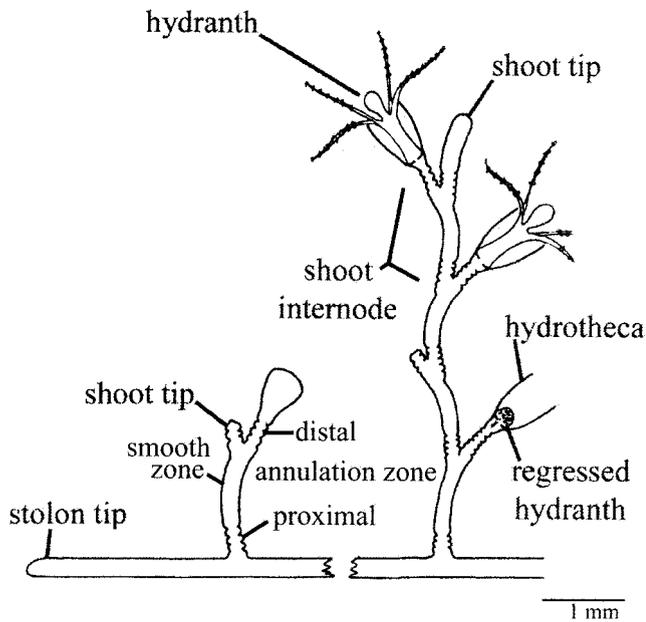


Figure 1. A colony of *Laomedea flexuosa* illustrating the perisarc and two hydranths.

cause a differential curving of the tip surface, and that this pattern is simply fixed by the perisarc. In this case the time at which the perisarc hardens has no influence on the shape of the perisarc. But shaping could also involve a differential pattern in time and space of perisarc hardening. When the perisarc hardens closer to the apex, the diameter of the growing tube should decrease thereafter. But when hardening takes place more distantly, the diameter can increase due to the tendency of the tissue in the apex to expand.

To test whether a differential hardening of the perisarc could play a role in the process of shaping, we treated the colonies with substances that could be expected to either support or to antagonize the hardening process. The effects we observed indicate that both a spatial and temporal pattern of perisarc hardening is involved in the shaping of the perisarc.

Materials and Methods

Animals

Colonies of *Laomedea flexuosa* Hincks (Thecaphora, Campanulariidae) were cultured on glass microscope slides in artificial seawater (Tropic Marine, 1000 mOsmol, pH 8.2–8.3) in a 5-l aquarium at 18 °C. The animals were fed daily with *Artemia salina* nauplii.

Test system

Shoots with newly emerged tips were used as test systems. Shoots including 4 to 6 distal internodes were isolated from the colony 2–4 h after feeding. The pieces were used immediately.

The treatment was performed in 4-ml petri dishes. Normally, the treatment lasted for 14 to 36 h. Under such conditions the shoot tip completed the formation of the internode in about 20 to 24 h. The animals were not fed during the course of the experiment. However, the tips of fed and unfed specimens grew with the same speed (Kossevitch, 1991). The medium was not changed. The results were scored at different times, starting 14 h after the beginning of the treatment. Measurements were made by means of an ocular micrometer. The proximal internodes of the isolated shoots that had completed their development before the start of the experiment served as the control, and were termed untreated.

Chemicals used for treatments

The stock solutions of the following chemicals were prepared in distilled water: 10 mM/l dopamine (Sigma), 10 mM/l *N*-acetyldopamine (Sigma), 5 mM/l phenylthiourea (Sigma), 0.1% Calcofluor white (Fluorescent Brightener 28 [Sigma]). The following stock solutions were prepared in seawater: 10 mM/l L-cysteine (Sigma), adjusted to pH 8.2–8.3, each time freshly prepared; staining solution for phenol compounds with fast red salt (Chroma, Stuttgart) according to Romeis (Clara, after Romeis, 1968), treatment for 3 to 5 min under visual control; 0.001% Congo red (Merck) and Evans blue (Merck), treatment for 5 to 15 min under visual control; 4% formaldehyde (Merck), treatment for 24 h.

Statistics

The significance of differences between data obtained following the various treatments was calculated by means of the *F*-test and the one-tailed *t* test.

Results

Architecture of the stolon tip and the shoot tip

The stolon and the shoot tip differ in size. During formation of the smooth part of a shoot, the tissue tube in the tip is about 160–250 μm in diameter and is in tight contact with the perisarc over a length of 250–350 μm (the perisarc is translucent). The tissue tube in the stolon tip is 200–300 μm in diameter and is in tight contact with the perisarc over a length of 300–500 μm . In both cases, adjacent to that region the tissue tube is much smaller in diameter and has tight contact with the perisarc at only a few positions.

The mode of perisarc formation

The composition of the perisarc is not well known but includes chitin and proteins (Jeuniaux, 1963; Chapman, 1973). The proteins of related species were found to contain a high concentration of disulfide bonds (Chapman, 1937; Bouillon and Levi, 1971). Phenol compounds are expected

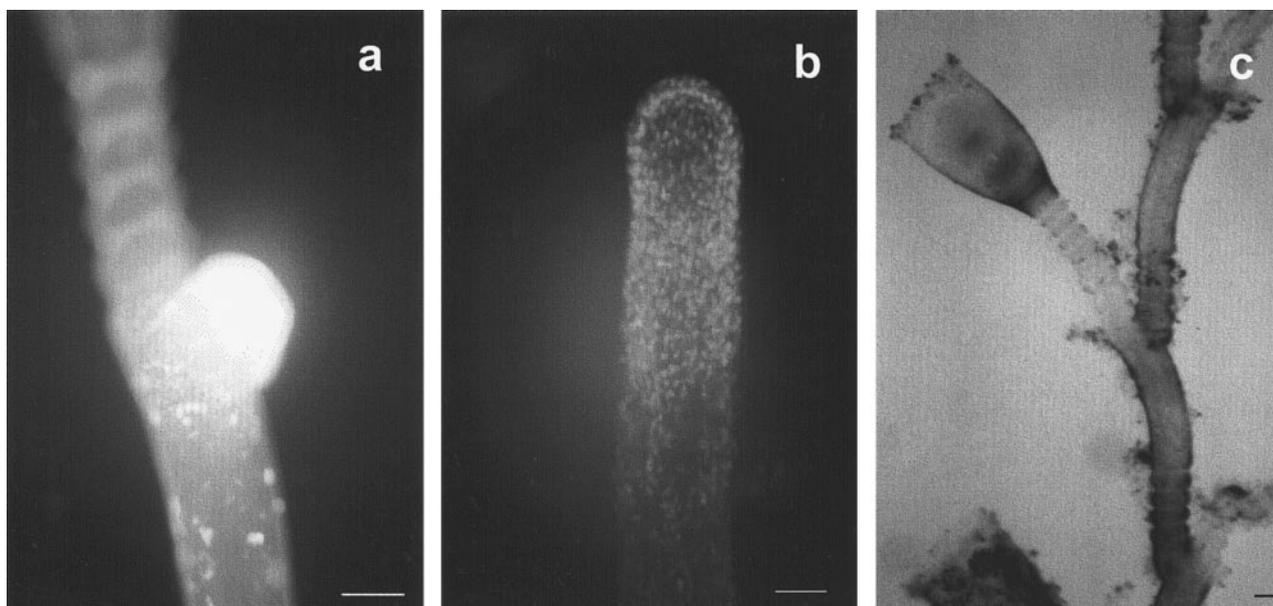


Figure 2. *Laomedea flexuosa* stained with various compounds. (a) A shoot stained with Calcofluor white. Note that the new tip is strongly stained, but the staining in the previously formed internode occurs in patches. (b) A stolon tip stained with formaldehyde. Note the decrease in the brightness of the fluorescence in the proximal direction. (c) Shoot perisarc stained with 1mM of dopamine. The proximal part of the internode and of the hydrotheca are well stained, but the distal annulation zone is almost unstained. The scale bar represents 100 μm .

to play a role in the hardening by causing a crosslinking between the proteins and the chitin (Knight, 1970).

Figure 2a shows the result of staining with Calcofluor white, which stains various carbohydrate fibrils, including amorphous chitin (Compère, 1996). The treatment stains the perisarc of the tip and in particular the outer surface of all ectodermal cells in the tip, that is, in the region in which all ectodermal cells contact the perisarc. Proximal to the tip, the ectoderm is not in close contact with the perisarc. In this region, the surface of the ectoderm, staining is observed to be in the shape of patches. The diameter of such a patch corresponds to the diameter of one or several ectodermal cells. The perisarc in the proximal part shows very little staining. No correlation between the spatial pattern of stained cells and the perisarc pattern could be detected. It appears that perisarc material is almost continuously secreted by the epithelial cells along the whole shoot, with the cells in the tip being the most active ones. That correlates with the finding that in old parts of the colony the perisarc is thicker than in younger parts. For example, the thickness of the perisarc wall in the smooth part of the internodes was found to change from proximal (the eldest) towards distal (the youngest) as follows (in μm): 11.95–8.34–7.56–6.10–4.39–3.17. Note that the distal part is stained but the proximal is not.

After formaldehyde was applied, a fluorescent stain appeared in cells of the ectoderm or at their surface. The stained cells were more numerous within the tip, but were

also found in smaller numbers along the whole tissue proximal to the tip (Fig. 2b). This result may indicate the presence of phenol compounds, which are known to play a role in the hardening process or sclerotization of the chitin-containing exoskeletons of various animals, including cnidarians (Knight, 1968, 1970; Holl *et al.*, 1992).

Although the perisarc looks almost uniform within an internode, it is not. Treatment with dyes including dopamine, fast red salt, Evans blue, and Congo red revealed a distinct pattern of staining of the perisarc. The most intense and spatially different staining was obtained with dopamine (Fig. 2c). The staining intensity decreases gradually from the most proximal position to the distal end of the smooth part. The distal annulated zone is not stained, whereas in the hydrotheca the staining is intense again. In elder internodes the pattern is identical, but the staining is *deeper*. Thus, the pattern of staining does not correspond simply to the thickness of the perisarc wall. Because of the chemical nature of the various agents and their binding specificity, we argue that these substances bind to phenol compounds, which may have played a role in cross-linking the proteins and the chitin in the skeleton (*cf.* Holl *et al.*, 1992).

The influence of L-cysteine on shoot patterning

L-cysteine is able to interfere with the formation of disulfide bonds between and within proteins. Thus, the application of L-cysteine may antagonize perisarc hardening if

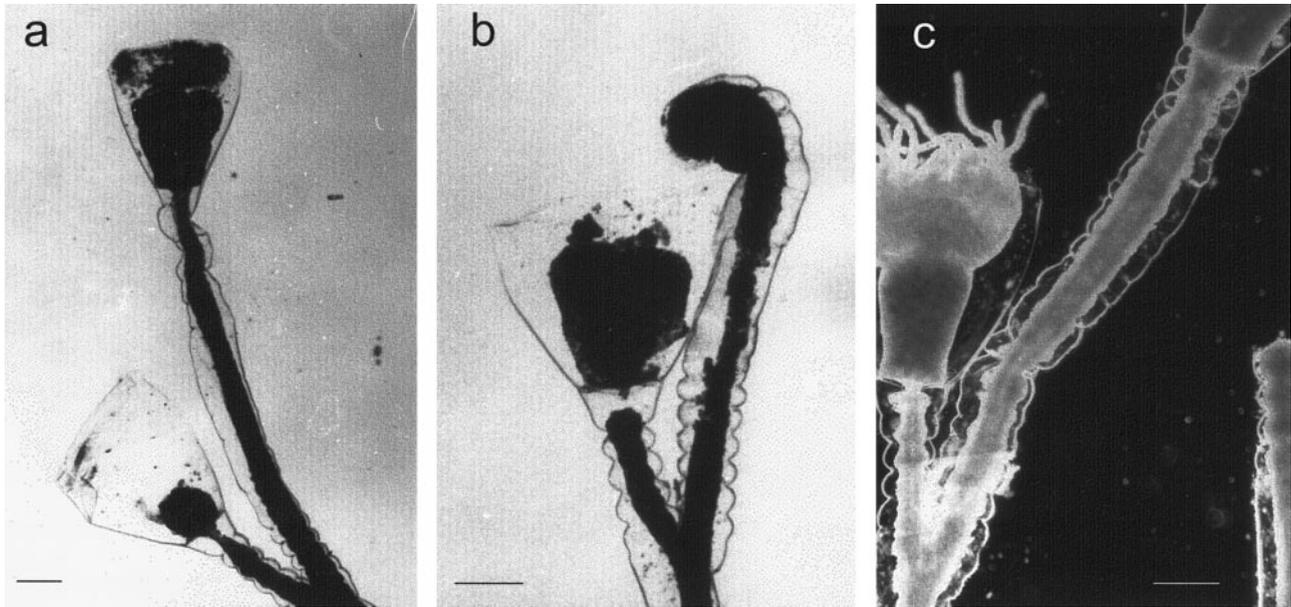


Figure 3. Alteration of the shoot perisarc shape due to treatment with L-cysteine and phenylthiourea. Treatment with 1 mM/l of L-cysteine. (a); 2 mM/l of L-cysteine (b); 0.25 mM/l of phenylthiourea (c). Note that the proximal annulation zone is smoothed, the smooth part is crumpled, and the distal annulation zone is smoothed and widened. Compare the normal pattern elements on the left side of each graph. The scale bar represents 100 μm .

the formation of disulfide bonds is involved in this process. In addition, L-cysteine impedes the formation of diphenols (Horowitz *et al.*, 1970). Diphenolic compounds including dopamine and *N*-acetyldopamine were shown to be involved in the sclerotization of the cuticle of insects (Kramer *et al.*, 1987; Sugumaran, 1987).

Treatment of shoots with L-cysteine greatly altered the shape of the perisarc. The perisarc tube widened, crumpled, and displayed folds at unusual positions (Fig. 3a, b). The smooth part and the distal annulated zone were especially affected. Most important, the annuli of the distal part, which form after the onset of the treatment, were not separated by the usual deep indentations, but displayed a much smoother pattern. (Compare as internal control the old pattern elements that formed before the start of treatment [Fig. 3]). The effect was observed following application of up to 1–2 mM/l of L-cysteine. Concentrations ten times higher caused the tissue to disintegrate.

Although the shape of the perisarc was altered to a great degree, the sequence of the pattern elements—such as the proximal annulated zone, the smooth part, the distal annulated zone, and the hydrotheca—was laid down as usual. It appears that even the volume of these elements was not significantly changed. Thus, the applied concentrations of L-cysteine did not strongly affect the pattern-forming processes in the tissue, but rather adversely affected the normal perisarc hardening. Due to the L-cysteine treatment, the perisarc remained soft for a longer period of time, allowing

external and internal mechanical forces to produce the observed malformations.

The influence of phenylthiourea on stolon and shoot patterning

Phenylthiourea, due to its sulfhydryl moiety, was also expected to interfere with the hardening of the perisarc. As was found for L-cysteine, phenylthiourea hinders the formation of diphenols by interaction with the monophenol monooxygenases (Lerch, 1983). Treatment of shoots by application of 0.25–0.5 mM/l of phenylthiourea resulted in the formation of bent and crumpled pattern elements. In particular, the distal annulated zone and the smooth part of the shoot were affected (Fig. 3c). Following treatment with L-cysteine, the annuli were not separated by the usual deep indentations but displayed a much smoother pattern. The sequence of pattern elements was unchanged.

Dopamine

The diphenol dopamine is an intermediate on the way to those diphenols that are involved in cross-linking of components of the cuticle in insects. In *L. flexuosa* Knight (1970) found dopamine and a phenoloxidase. He suggested that both substances generate quinones that react to cross-link structural proteins. We found that 0.1 mM/l of dopamine reduced the maximal diameter of both the smooth part and the distal annulated zone. At the same time, the length

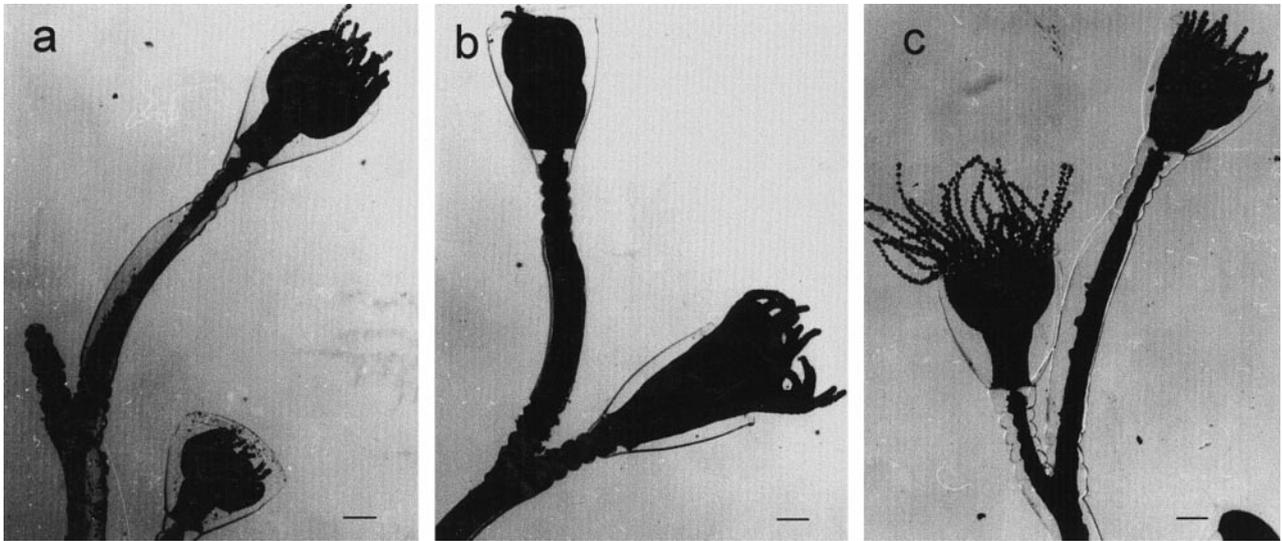


Figure 4. Alteration of the shoot perisarc shape due to treatment with dopamine and *N*-acetyldopamine. (a) Treatment with 0.1 mM/l of dopamine (b) Untreated control (c) Treatment with 0.1 mM/l of *N*-acetyldopamine. Note that the smooth part and the distal annulation zone are increased in length, and the proximal annulation zone is irregular in shape due to treatment with *N*-acetyldopamine. Compare Fig. 4b as control. The scale bar represents 100 μ m.

of these pattern elements increased (Fig. 4a, Fig. 5). In the annulated zone, the ratio between the maximal outer diameter of the annuli and the diameter of the furrow between adjacent annuli remained almost unchanged (not shown). In the proximal annulated zone, the effect was less pronounced. One reason may be the short interval between the onset of treatment and the formation of the proximal annuli. Further, the composition of the perisarc may play a role. The resultant staining of the perisarc was strong in the

proximal annulated zone and almost absent in the distal annulated zone (Fig. 2c).

N-acetyldopamine

In insects, *N*-acetyldopamine is thought to be an intermediate between dopamine and the diphenols used for cross-linking of the cuticle (Kramer *et al.*, 1987; Sugumaran, 1987). Knight (1970), however, suggested that the mecha-

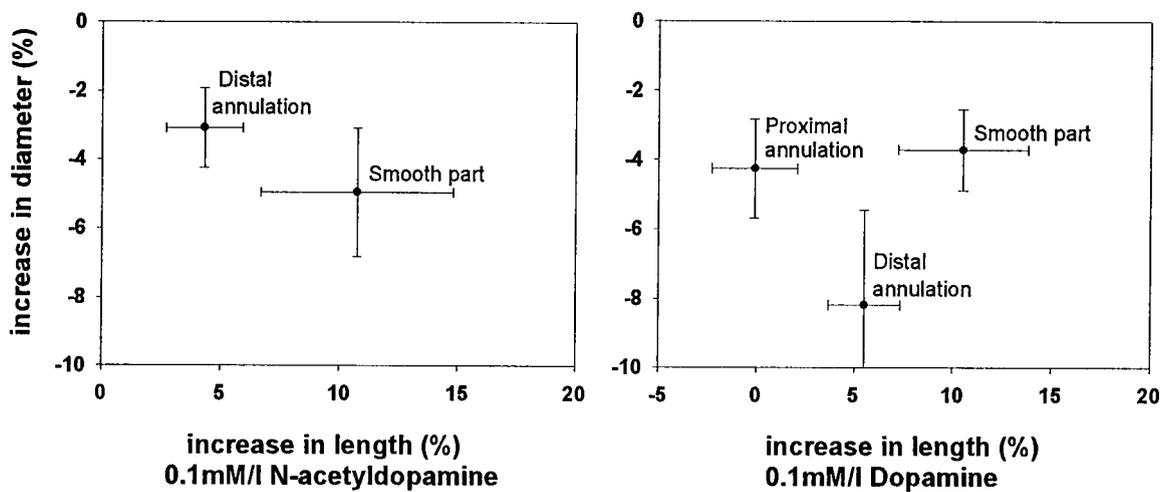


Figure 5. Treatments of growing tips with dopamine and *N*-acetyldopamine change the diameter-to-length ratio of the structural elements of a shoot internode. A concentration of 0.1 mM/l of the compounds was applied. The graphs show the changes in the size (dimensions) of the internode parts. The data are given as percent of increase as compared to the respective control, the previously formed internode. The bars indicate the standard deviation of the mean, dopamine, two experiments, $n = 9, 10$, and *N*-acetyldopamine $n = 7$.

nism of sclerotization of the hydroid perisarc differs from that of insects, because he failed to detect *N*-acetyldopamine and phenolic- β -glucosides in hydroids. In *L. flexuosa*, a concentration of 0.1 mM/l of *N*-acetyldopamine caused the smooth part and the annuli of the distal annulated zone to become narrower and longer (Figs. 4a, b; Fig. 5). Further, as observed for the treatment with dopamine, the ratio between the maximal outer diameter of the annuli and the diameter of the furrow between adjacent annuli remained almost unchanged. Unlike dopamine, *N*-acetyldopamine strongly affected the proximal annulated zone, eliminating its regular annulation pattern (Fig. 4c). *N*-acetyldopamine may act faster than dopamine.

There is no indication of an unspecific, cytotoxic action of the chemicals. One can see in the figures that the older colony elements are unaffected by the treatment: polyps stretch out of their hydrotheca, and they are able to catch their prey. The hydrothecae formed during treatment with the chemicals are well shaped, and living polyps formed with tentacles.

Discussion

The delicate species-specific pattern of a thecate colony is laid down exclusively at the growing tip. At this site, the tissue has permanent contact with the expelled soft material from which the outer skeleton, the perisarc, is formed. Some dozen micrometers proximal to the apex of the tip, the perisarc hardens, which fixes the pattern of the perisarc.

It is obvious that the soft material is molded by the outer shape of the underlying tissue. This outer shape is determined by the property and activity of the cells that built the tissue tube, particularly those cells that produce the growing tip. In the tip, the tissue moves back and forth rhythmically. This phenomenon, termed growth pulsation, has been studied extensively (Belousov *et al.*, 1992).

The staining with Calcofluor white suggests that the amorphous perisarc material that eventually forms fibrils, including chitin fibrils, is secreted by almost all the ectodermal epithelial cells of the growing tip, as well as by some epithelial cells along the body axis. The phenolic compounds, which Knight contended to be involved in the cross-linking of the perisarc, appear to be contained in so-called tanning cells (Knight, 1970). These cells are concentrated in the tip and also exist in lower density in the proximal parts. They have no broad contact with the outer surface of the epithelial sheet of the growing tip and are embedded between the epithelial cells (Knight, 1970).

Our data suggest that a differential hardening of the perisarc is involved in the shaping of the perisarc tube. We treated a growing culture with substances that we expected, from their chemical nature, to affect the hardening process. Phenylthiourea and L-cysteine were expected to impede the hardening; dopamine and *N*-acetyldopamine were expected

to support it. The putative "softeners" caused a crumbling and a widening of the perisarc. Of particular importance is that the constrictions between the annuli were smoothed out in the distal annulated zone. The putative "hardeners" caused the perisarc tube in all internode parts to become narrower and longer. The applied concentration of the various chemicals was apparently not toxic to the animals: in the presence of the chemicals the polyp and the hydrotheca of the internode formed normally and the polyps behaved normally—for example, in stretching out to catch their prey.

We suggest that the composition of the soft perisarc material surrounding the apex changes with time. The nature of the compounds is largely unknown. In insects, low-molecular-weight catechols such as *N*-acetyldopamine and *N*- β -alanyldopamine are involved in sclerotization. These are converted to quinones, which react in cross-linking proteins (for general review see Waite, 1990). Knight (1970) proposed a different mechanism of action for *Laomedea flexuosa*: failing to detect the mentioned substances, he detected dopamine instead, and suggested that it was active in sclerotization. Waite (1990) stated that "this should be taken with caution since the entire animal was methanol-extracted." In organisms other than insects, dopa-containing proteins are thought to cause the sclerotization through a process of "autotanning" (Smyth, 1954; Brown, 1952; Pryor, 1962) in which the dopa moieties are converted to quinones. Additional molecules—of chitin, collagen, fibroin, or cellulose, for example—are necessary as "fillers." This mode of sclerotization is well-distributed throughout the animal kingdom, and Waite and coworkers (1990) found dopa-containing proteins in the cnidarian *Pachycerianthus fimbriatus*. Our results do not help resolve the question of which mode of sclerotization acts in *L. flexuosa*. We know, however, that the concentration of one or several of the components changes rhythmically during the growth of the shoot internode. These rhythms are much slower than those of the aforementioned growth pulsations. If the hardening occurs closer to the apex, the diameter of the ring-shaped border between the hard and the soft perisarc decreases, forcing the tissue to squeeze through this opening. Under these conditions, the perisarc tube elongates with a reduced diameter. A widening of the diameter needs at least two prerequisites: the hardening has to happen more distally from the apex than before, and the tissue of the apex must form a bulb. Evidence for bulb formation may be that the tissue tube in both the tip of the stolon and the tip of the shoot has a tight contact to the perisarc, while in proximal regions the tissue tube is much smaller than the inner lumen of the perisarc tube. Further, the shoot and the stolon occasionally form a bulb at the wound after cutting (Kossevitch, unpubl. obs.).

In the process of annulus formation, the zone of hardening may move rhythmically closer to and then farther away from the apex. This may occur in either a continuous or a

stepwise manner. When a hydrotheca starts to form, the zone of hardening lags behind in relation to the apex of the protruding tissue. That causes a widening of the tissue tube and subsequently of the perisarc tube as well.

In *L. flexuosa*, the observed bending of the tube in the smooth part of the internode (cf. Fig. 1) may be the result of an asymmetry in the hardening of the perisarc along the circumference of the tip. It may occur closer to the tip apex at the side that faces the shoot axis, imposing a spatial control of hardening in addition to the temporal control.

In other animals with an exoskeleton, such as arthropods, the integument may be shaped by changes in the hardening process together with changes in the pressure of the tissue against the forming integument. In arthropods other than thecate hydrozoa, the hardening can start at various positions and can spread at different speeds from those positions. The resultant shape of the integument can thus be more complex than in hydrozoa.

The various treatments we applied caused the perisarc to bend, to fold, and to crumble. However, the sequence of the pattern elements up to hydrotheca formation was as normal as possible. The volume of the tissue responsible for the formation of the corresponding element was largely unchanged. The decrease in the diameter of the perisarc tube was compensated for by the elongation of the tube. This indicates that the very tip determines the sequence of pattern elements. The respective decisions of the tip were not influenced by (1) the chemicals applied in the concentrations noted, (2) the disturbance of the shape and movements of the tissue in the tip, (3) the shape of the tissue tube in a more proximal region, nor (4) the altered tension and pressure of the proximal tissue on the tissue in the very tip. These four points are in agreement with the observation that the experimentally isolated shoot tip continued the patterning program of the perisarc tube up to the formation of the polyp's housing. The tissue itself was transformed into only the apical part of the polyp; the proximal part of the perisarc tube was free of tissue (Kosevich, 1991).

Acknowledgments

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