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Histological peculiarities of the noding process in *Cyprideis torosa* (Jones) (Crustacea, Ostracoda)

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Abstract

The noding of *Cyprideis torosa* is investigated. Studies reveal structural changes in the ornamentation and the cellular layers of the epidermis in the noded area. Noding is caused by the inability of the animal to regulate the increasing osmotic pressure during moulting in low saline water. Therefore it must be considered as a phenotypic and not a genetic response.

Introduction

Cyprideis torosa (Jones, 1850) is a member of the brackish biocoenosis in European coastal waters. It belongs to the small crustaceans classified under the name Ostracoda, characterized by a calcified shell which encloses the body and appendages, and by a moulting process involving the already shelled nauplius and eight stages up to the adult.

C. torosa is very abundant in hypo as well as in hyperhaline waters. It seems to have developed two methods of osmolarity adjustment, namely an amphiosmotic and a confohyperosmotic regulation (Aladin, 1987). In very low marine conditions (<5%salinity) it develops nodes at certain places on the shell (Sandberg, 1964; Vesper, 1972). Noding is used widely in palaeontology as an indicator for brackish water environments.

Several attempts have been made to clarify the origin of these nodal structures (Fassbinder, 1912; Müller, 1912; Triebel, 1941; Hartmann, 1964; Sandberg, 1964; Jorgensen, 1970; Kilenyi, 1972; Vesper, 1972; Van Harten, 2000). However, until now it is not clear whether this structure is genetically induced, or whether salinity and other environmental factors initiate the noding response. A clarification would also help to assess the taxonomical importance of noding in other mainly freshwater ostracod species of the genera *Limnocythere* and *Ilyocypris*. The present study tries to find answers to this problem.

Materials and methods

Ostracods were collected using a hand net with 180 μm mesh and picked one by one with a pipette under a stereomicroscope. These animals were kept alive in small bowls with water of different salinity at 12–15 °C and 14 h of light each day, with a weekly change of water and no extra food.

Living animals were observed with a stereomicroscope for special behaviour and to spot the right time for fixation. They were fixed in 70% ethanol, dissected with needles and embedded and stained in polyvinyl-lactophenol with Orange-G. Some animals were macerated by heating in KOH (20%) prior to dissection and then embedded. Others were put in clove oil for a fortnight to make them translucent and then observed under the microscope.

Specimens used for sections in the light microscope (LM) and in the transmission electron

microscope (TEM) were treated in basically the same way. They were fixed in 2.5% glutardialdehyde in 0.05 mM phosphate buffer containing 5% sucrose. Then they were washed three times in buffer with sucrose, post-fixed with 2% osmium tetra oxide in the same buffer, washed again three times and decalcified in 0.1 M EDTA for 12 h. After that they were dehydrated in a graded row of acetone before being embedded in Spurr's resin (Spurr, 1969) and cured for 24 h at 60 °C.

Semi-thin and ultra-thin sections were cut at the REICHERT Ultracut E Ultramicrotome. Semi-thin sections were stained with toluidine blue and pyronine after Holstein & Wulfhenkel (1971). Ultra-thin sections were stained with uranyl acetate (Stemper & Ward, 1964) and lead citrate (Reynolds, 1963). Photographs were made on a LEITZ DIALUX for LM and on a ZEISS EM 902 for TEM.

Specimens for the scanning electron microscope (SEM) were fixed in 2.5% glutardialdehyde in phosphate buffer, dehydrated in a graded acetone series and dried (critical-point) with CO_2 in a BALZERS CPD. They were sputtered in a GEA-004 S manufactured in Graz, Austria, and viewed under a CamScan SEM DV 4 and a LEO 1525.

Cryofixed animals were sectioned at −150 °C on the REICHERT Ultracut E Ultramicrotome equipped with the FC4 Cryostage. The animals were dehydrated at −80 °C in a cooler in 100% acetone for 4 days, and then warmed up to room temperature and critical point dried.

Results

General structure of the carapace

The carapace of an ostracod consists of different living and excreted layers. To understand the construction, one has to realize that primarily a folding of the dorsal part of the animal occurred, producing a sac-like extension. This sac encased the whole body and developed a sharp edge at its ventral tip. Additionally, a furrow, which is called the hinge, developed in the middle of the dorsum to enable the lateral parts to move up and down. The development of the part of the sac facing the exterior and the part facing the body is different. The outer part is encrusted with calcite and fairly thick, while the inner part remains as a thin cuticle. Both of these layers are excreted by a thin layer of living cells, the epidermal layer, which lines the inner part of the whole sac-like extrusion (Fig. 1).

To stabilize these layers, the epidermal cells of the inner and the outer lamellae are connected by cell-to-cell contacts, which minimize the free space between the epidermal linings. The animal uses this space in different ways. It accommodates the hepatopancreas tubes, testicle tubes or ovaries, nerves run through it and the body liquid is also pumped through it. The inner cuticle lining is thin enough to permit the uptake of oxygen and at the same time to excrete water for osmolarity control (Fig. 2).

The area where the inner lamella is connected to the body itself is called the isthmus. Here the connection between the inner epidermal cell layer and the outer epidermal cell layer is lost and the inner cell lining is connected to the body cuticle. The outer epidermal cell layer continues to the hinge and from there to the other side of the animal where it is again connected to the inner epidermal cell layer on the other side (Fig. 3).

Animals with a hard exocuticle have to get rid of the cuticle in order to grow. This process is called ecdysis or moulting. There are several processes associated with shedding of the old exoskeleton. First, the epidermal cells produce a substance which separates the outer cell membrane from the cuticle. Second, a new and flexible cuticle is secreted. This cuticle is folded so as to be larger than the old one after expansion. In order to shed the old cuticle, the animal changes its volume by raising the osmolarity of the body fluid thereby increasing water uptake. This rise in the volume of body fluid causes the old cuticle to tear along pre-formed edges. The animal moves out of the old encasement by stretching and enlarging the new cuticle. Calcification as in ostracods then follows (Fig. 4).

During this whole process, the epidermal cell layers go through a variety of different stages. As is demonsrated in the present study, the process of noding in *Cyprideis* is strongly connected to moulting.

Histology of the nodes

Noded animals were collected mainly around the coast of the Baltic Sea at salinities between 15 and 2‰. The amount of noded specimens increased with lower salinity, although at any given salinity

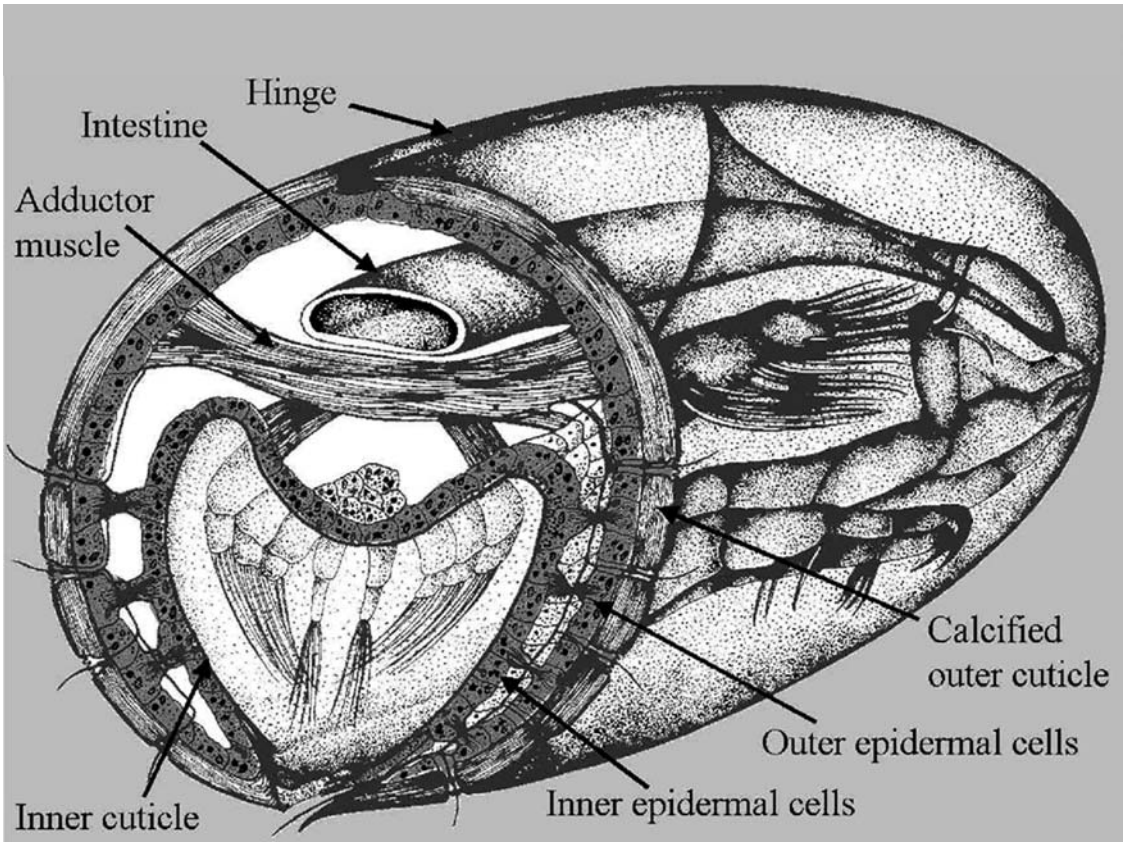

Figure 1. Schematic section of an ostracod (perpendicular section proximal to the adductor muscle scars) showing the organisation of the shell.

animals with and without nodes were found. Reference samples from the North Sea coast showed only slight noding in very low salinity, around 2–3‰. The noding of both shells is often asymmetrical; one side can be nearly smooth while the other side is nodose (Triebel, 1941; Vesper, 1972) (Fig. 5).

SEM pictures show that nodes in *C. torosa* occur at distinct places on the lateral side of the carapace (Sandberg, 1964; Kilenyi, 1972). The immediate areas surrounding the nodes and the nodes themselves are obviously stretched as one can see very well in the strain lines of the mesh depressions (Fig. 6). The surface resembles an over-stretched rubber balloon that has lost some of its air again. Basically, however, the surface of the node is not different from the surface of other areas on the shell.

The nodes often have a varying appearance. They are usually round protrusions, but sometimes, especially in heavily noded specimens, they can extend enormously, especially when two normally separated nodes are connected (Fig. 6). Nodes may also coalesce into steps of different height. In this case they span the distance of several underlying epidermal cells.

The view from the inner side of the shell with the epidermal layer removed, as one finds them often in the sediment, shows a clean surface with hollows where the nodes are. One side of the hollow is always steeper than the other. A special structure in these hollows is not recognisable, at most, a slightly rougher surface might be present. The normal pore canals as seen from the inside are also not different from other parts of the shell.

The nodes in *C. torosa* develop only in places where the shell is not attached to the body of the animal, i.e. outside the isthmus, as stated by Sandberg (1964; Fig. 7). The main nodes that are found in the majority of noded specimens are al-

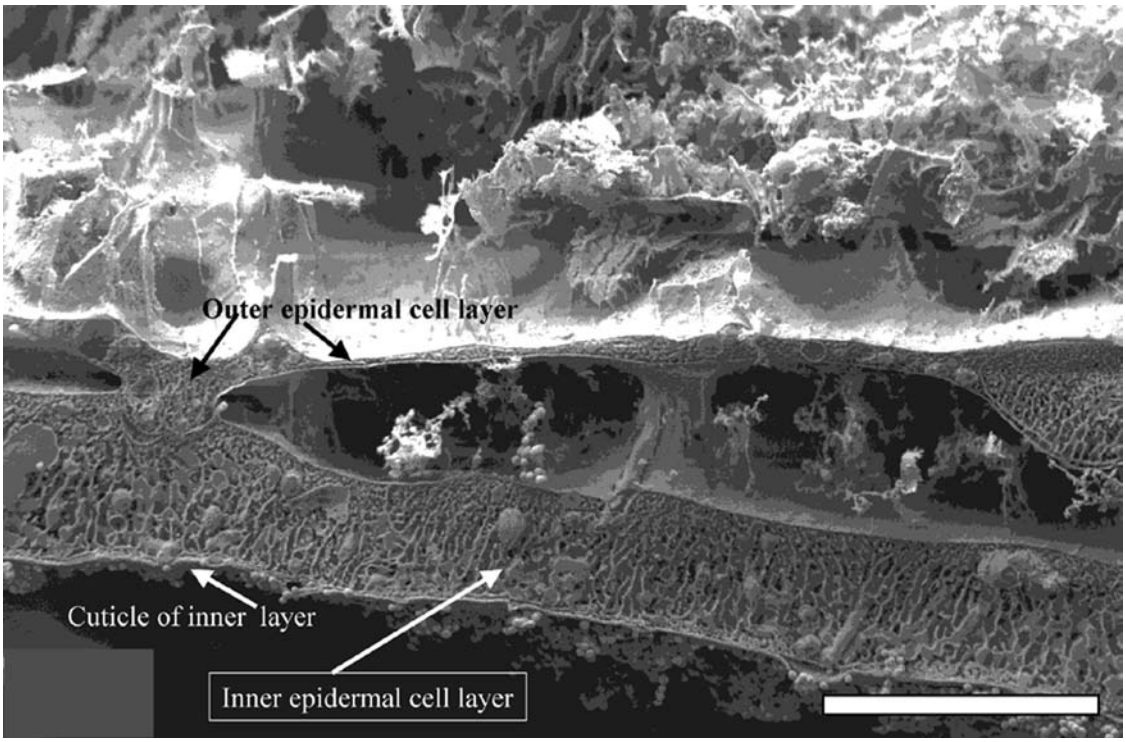

Figure 2. Scanning Electron Micrograph of fracture face through the shell, showing the organisation of the inner and outer epidermal cell layers (Scale bar = 10 μm).

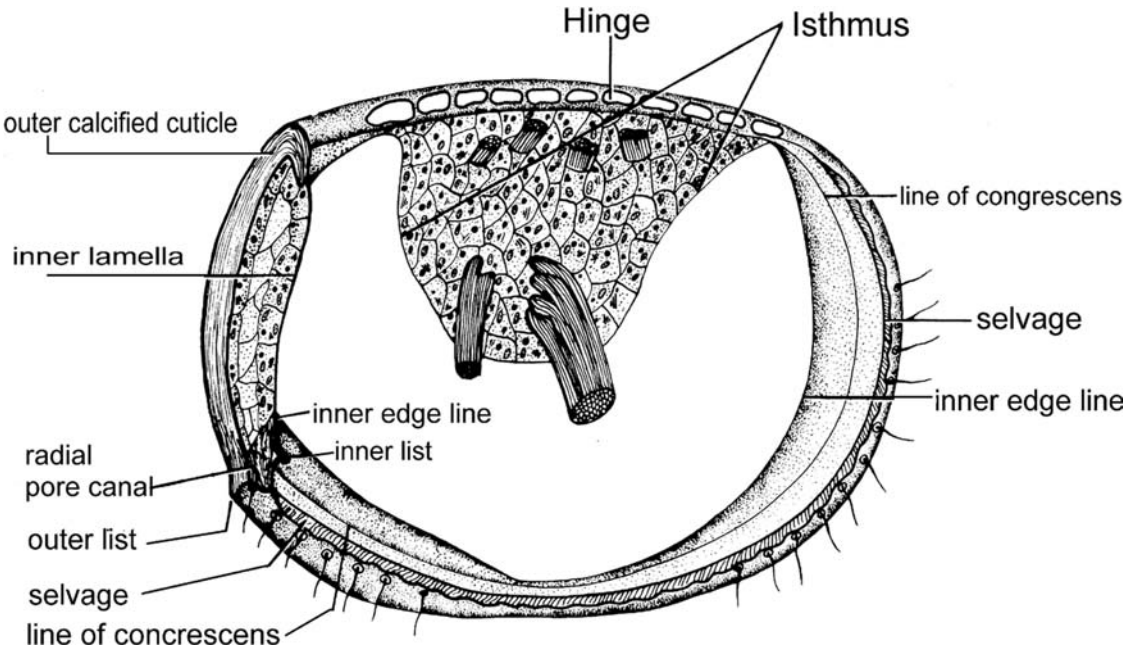

Figure 3. Schematic section of the inside of the ostracod shell showing the connection to the body.

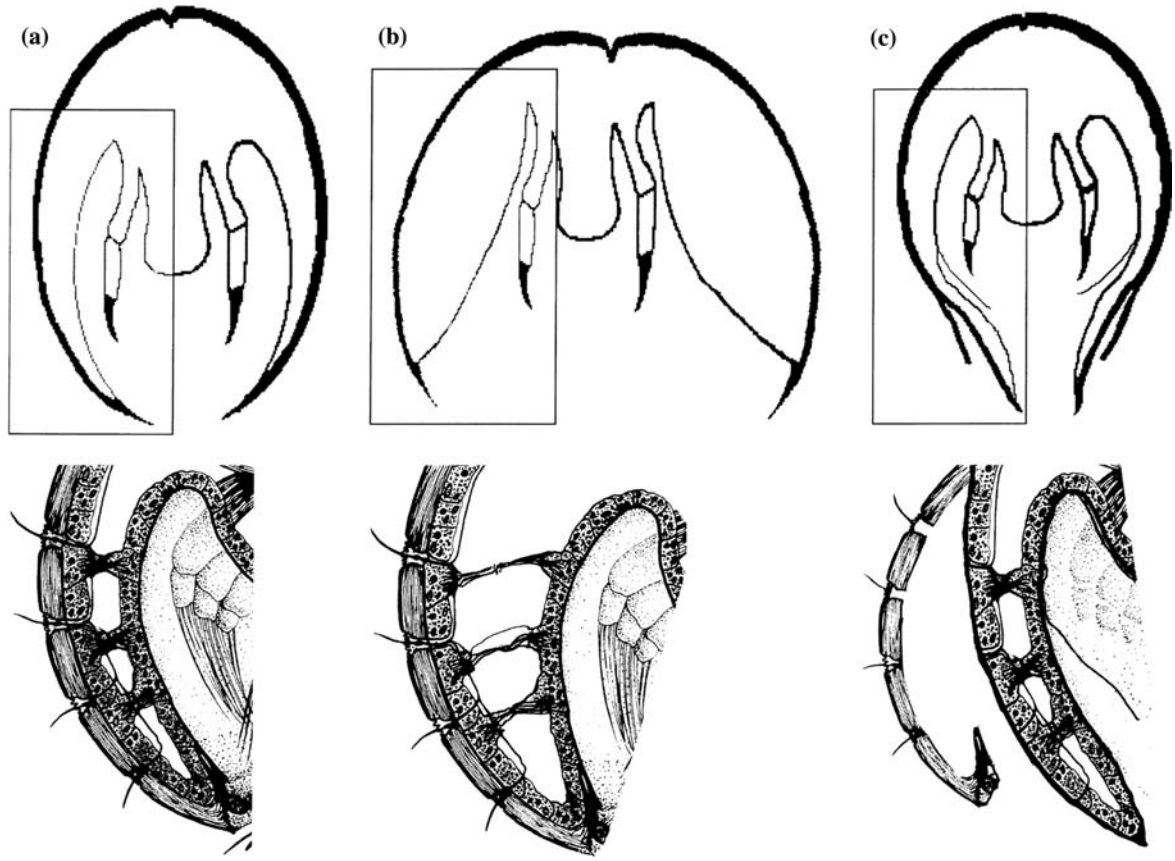

Figure 4. Schematic diagrams of different stages of the moulting process: (a) normal stage prior to moulting, (b) swelling stage just prior to rupture of the old cuticle, (c) relaxation stage, when the new cuticle has room enough to stretch out of the old cuticle. Insets: the state of the cell-to-cell connections in the carapace.

ways situated near the isthmus boundary. In some rare cases the nodes are so big that they change even the direction of the free margin in the animal (Fig. 5). However, this seems to have no effect on the activity of the animal.

Fractures of the shell in SEM show the organization of the different layers (Fig. 2). The inner epidermal cell layer is normally thicker than the outer, which is often only a thin sheet underlying the calcified cuticle. Underneath the nodes, the outer epidermal cells are remarkably thick, the space of the body cavity is enlarged and also the inner epidermal cells are sometimes larger. This is the reason why the outer epidermal layer extends into the cavity of the node, although the inner cuticle does not show any sign of an indentation. Histological sections through the carapace also show this.

Nodes are more strongly calcified than other parts of the shell (Kilenyi, 1972). In weakly calcified specimens, the amount of chitinous substance underlying the nodes is unusually high. It is a filamentous network made up of parallel sheets, which are secreted perpendicular to the cell membranes of the outer epidermal cell layer.

Sieve pores within the nodes are only seldom distorted; they act mostly as stabilizing structures in these parts of the cuticle.

In smooth specimens, the outer epidermal layer contains lots of small granules, which can also be found at the inner lining of the carapace, thus building the calcification of the shell (Rosenfeld, 1982). Noded specimens have fewer calcification granules.

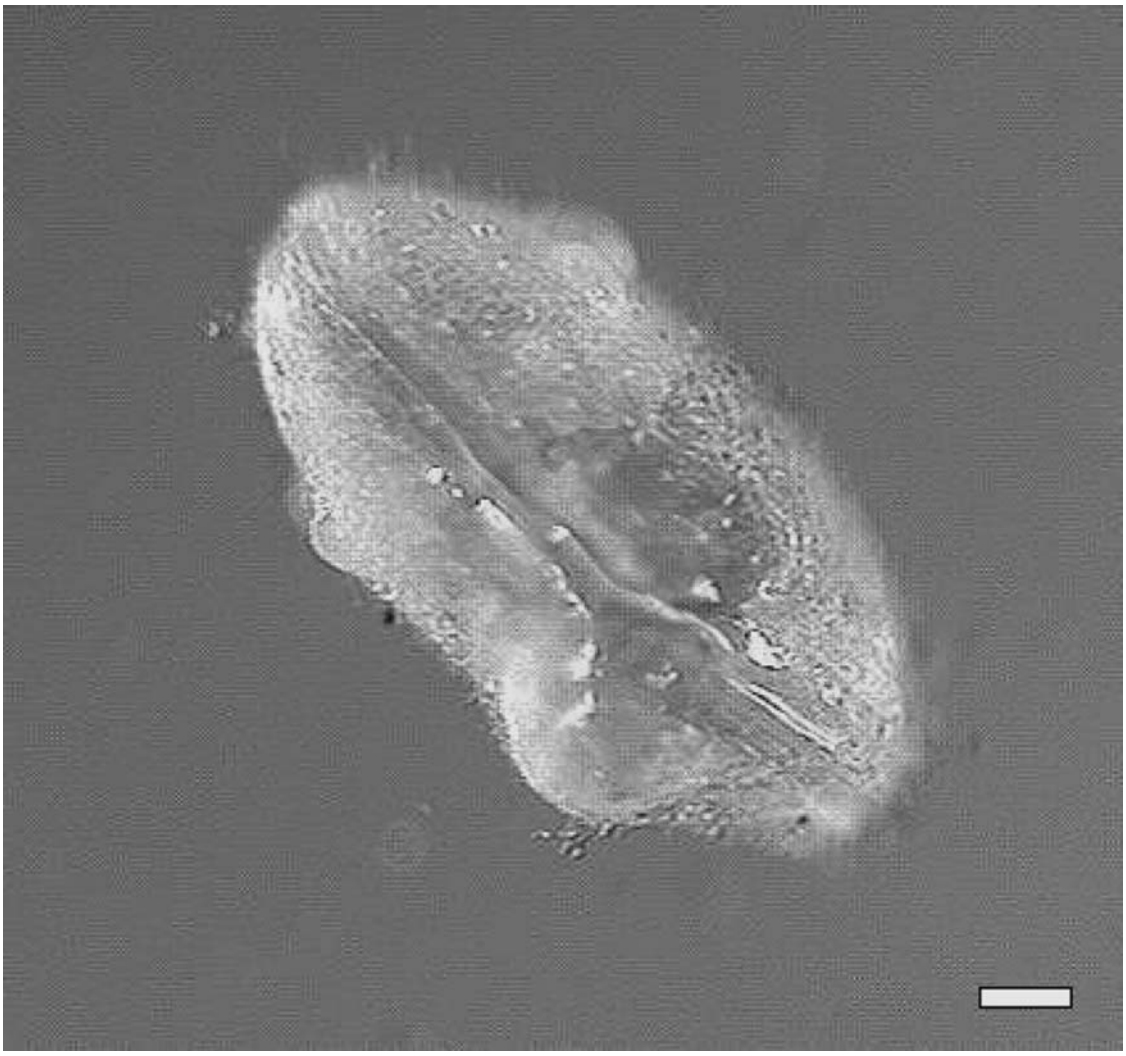

Figure 5. An asymmetrically noded *C. torosa* carapace (Scale bar = 100 μm).

Discussion

The process of noding has been interpreted in many ways in the literature. This question in particular was intensively discussed at the first Ostracod symposium in Naples in 1963. Kilenyi (1972) summarized all the ideas and theories. The main ideas were:

1) Nodes are genetically induced features of different species (Brady, 1868).
2) Nodes are hollow and reduce, therefore, the specific weight (Triebel, 1941).
3) Nodes are phenotypic and caused by low salinity. (Van den Bold, 1946; Wagner, 1957).
4) Nodes are phenotypic and caused by low calcium content (Hirschmann, 1912).
5) Nodes are of ecological – genetical origin (Kilenyi, 1972).

Since that time the nodes have been used widely in the literature but no real attempt has been made to clarify the causes of noding in *Cyprideis*. However, three aspects already published needed to be combined.

In 1972 Kilenyi stated, ''The organic integument must already possess the structures that after calcification will be the nodes. As calcification is a rapid process, it is rather difficult to believe that during this short period between moulting and the

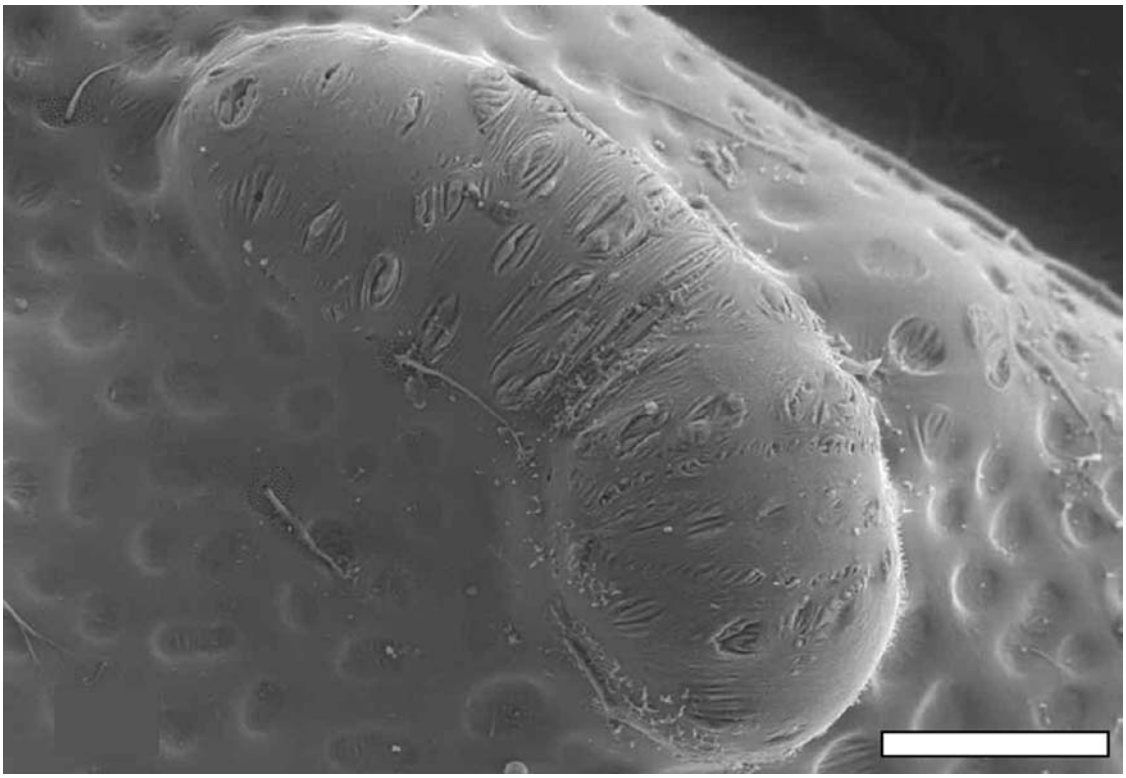

Figure 6. *C. torosa* larvae A-4; node on the ventral part of the left valve showing stress-related inflated and released mesh depressions on the noded part with strain lines (Scale bar = 50 μm).

appearance of the calcified shell some environmental factor could exert such an influence'' (Kilenyi, 1972). He also referred to the stable location of the nodes and therefore concluded that this was a genetically controlled structure.

Sandberg (1964) mentioned that most of the nodes are outside the isthmus (all in *Cyprideis*) and left it open whether it is a phenotypic or genetically controlled phenomenon.

Based on culture experiments, Van Harten (1996, 2000) concluded that noding is connected to the osmotic situation of the environment and called nodes ''a frozen memento of an accident of sorts that happened during the previous moulting'' (Van Harten, 2000: p. 133).

In this study details of the ornamentation of the nodes show that the cuticle is stretched and then released during moulting (Fig. 6). This means, that nodes must form prior to, or during moulting when the new, fully developed cuticle is flexible enough to allow such stretching and retraction. Therefore, to get a better idea of what causes noding, we have to study the processes during moulting.

Nodes occur only outside the isthmus of the shell, where the outer and inner epidermal cell layer are connected to each other. Consequently this is the area of interest (Fig. 7).

During moulting the animal raises the osmolarity in its body fluid and presses the liquid into the shell where the pressure disrupts the old cuticle along the inner edge of the calcified zone. The animal with the new cuticle crawls out of this rupture.

In low salinity the amount of osmotic active substances secreted into the body fluid must be lower than in high salinity water in order to have the same effect. In the case of *Cyprideis*, the amount seems to be higher than necessary when the outside salinity drops below 5% (Schäfer, 1953). This leads to higher pressure than normal when the animal presses the body fluid into the carapace. This pressure is applied into the caverns between the inner and outer epidermal cell layer causing this part to blow up like a small balloon. This affects mostly the inner part of the shell, for the cuticle there is thin and more flexible than the calcified outer cuticle.

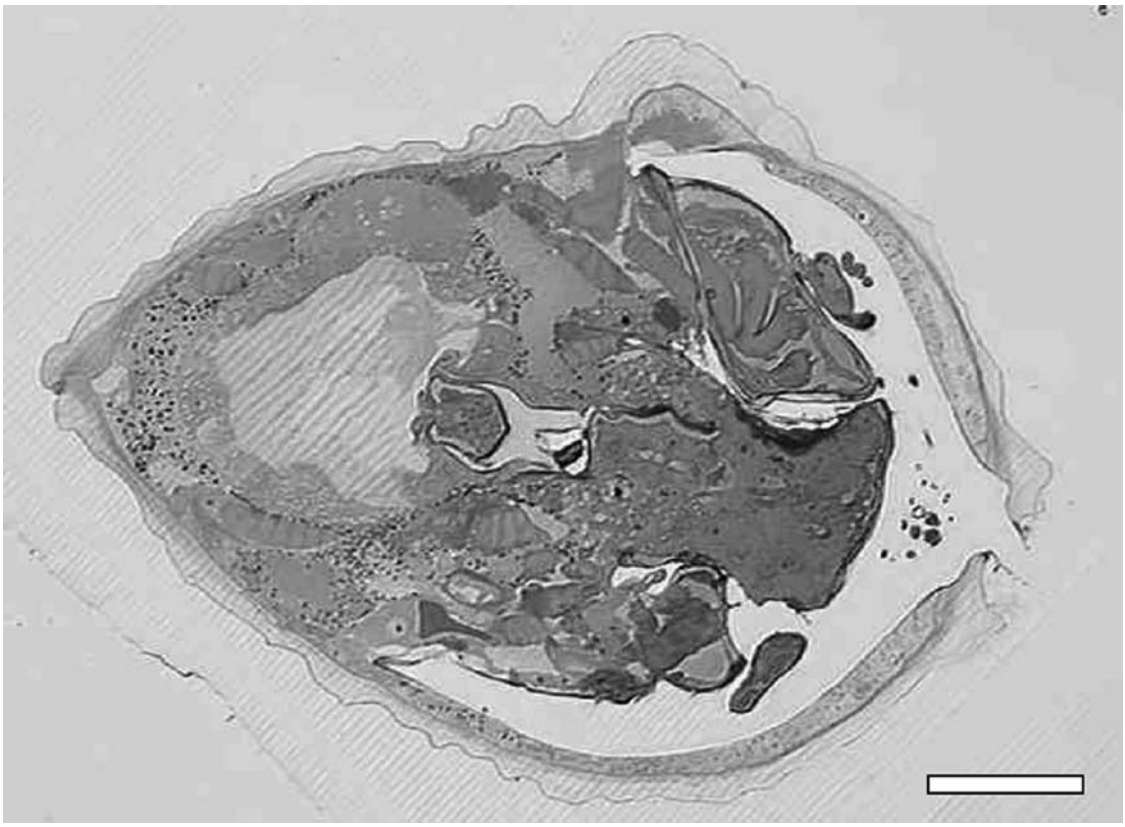

Figure 7. Section of *C. torosa* A-4 instar in the vicinity of the adductor muscle scars; note that the node is outside the isthmus area (Scale bar = 50 μm).

Only the small cell-to-cell connections between the inner and outer epidermal cell layer will resist this force. In some areas muscles adhering to the cuticle also help to withstand the pressure. These connections must be flexible enough to permit the expansion of the shell in order to tear the old cuticle at the preformed rupture line situated between the calcified inner lamella and the non-calcified inner lamella. But they also must be able to contract the shell again when this process is over.

During the expansion process just prior or during moulting, it is possible that some of these cell-to-cell connections tear in certain places and the new cuticle, flexible as it is, will give way for a small or big protrusion to emerge in this area. All pre-formed ornamentations on the new cuticle are stretched and will also keep their stretched appearance visible in many strain lines when the inflated carapace contracts. So, protrusions will be calcified and noded specimens will appear (Figures 5, 8 and 9).

Accordingly, the primary cause of noding is a failure to regulate the osmotic pressure inside the ostracod during moulting. This seems to happen more often when the salinity is lowered. The critical level where the majority of *C. torosa* have real difficulties to balance this is about 5%. Naturally, some individuals will always adjust better than others. This explains why it is always possible to find animals with and without nodes in one sample.

Why do the nodes develop at certain places on the shell? The force to tear the old cuticle is applied to the shell by means of the body fluid. The body fluid is distributed throughout the shell by a kind of weak circulatory system. This system is connected at certain places to the system of the body itself. Here the pressure is applied first and therefore these places are most likely to tear apart and give room for a node.

Why are the nodes different in size and height? The cell-to-cell junctions might rupture in one place,

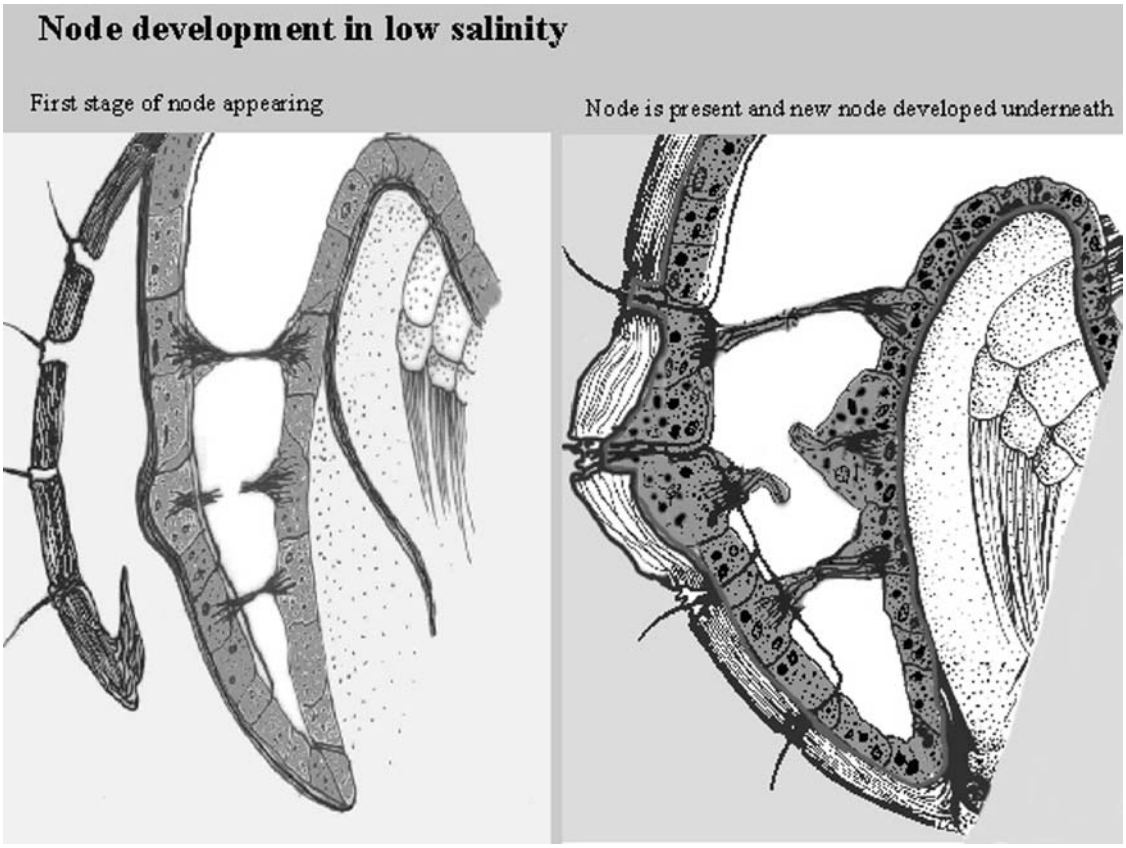

Figure 8. Process of noding with the cell-to-cell connection tearing.

or they might be pulled apart over a wider area, so the node is small or large, respectively. If the pressure is low, the protrusion will be low (Fig. 9).

Also the different development of the nodes on the left and right valves of some individuals is clarified. Moulting in *Cyprideis* takes place when the animal is lying on one side of the shell. The side facing the substrate needs more pressure to develop a node of the same height as the opposite shell that faces the water, for it, has also to lift up the weight of the body. In this case the body weight is an additional force that withstands the osmotic pressure in the shell at certain places. These conclusions resolve many questions in an unconstrained way.

However, it may well be that a second process is additionally connected to noding. The Ca content in lower salinity is also lower than in high saline waters. Muscle and desmosomal activities are strongly dependent on the presence of calcium ions. In the muscle it will change the configuration of the troponin molecule, so that myosin can be bound to the actin filaments. Calcium also has a comparable function in the configuration of desmocolin, desmoplakin and cadherin; all are molecules integrated in the desmosomal functions at cell membranes. These are necessary for the desmosomal junctions present between the outer and inner epidermal layer in *Cyprideis* (Fig. 10).

If there is a lack of free available calcium in the body fluid during the process of moulting, these junctions as well as some muscles will not be flexible enough and may tear more easily. This is comparable to a cramp in some muscles in humans (Fig. 11).

This additional fact might be responsible for the capability of *Cyprideis* not to develop nodes in Ca-rich surroundings in fairly low saline environment, as for instance the German North Sea coast with its clayish substrate.

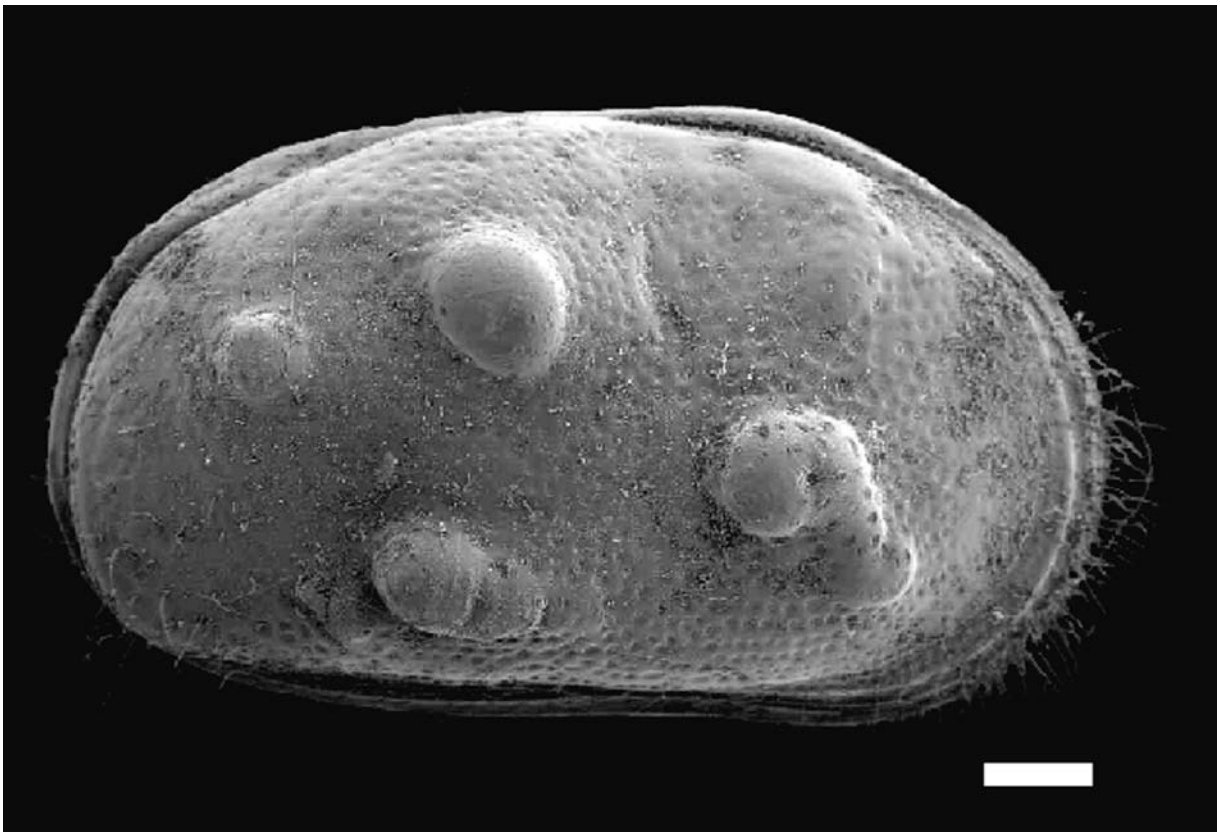

Figure 9. Adult *C. torosa* heavily noded; remarkable is the step-like protrusion on the ventral side and the S-form node in the front. Scale bar = 100 5 μm

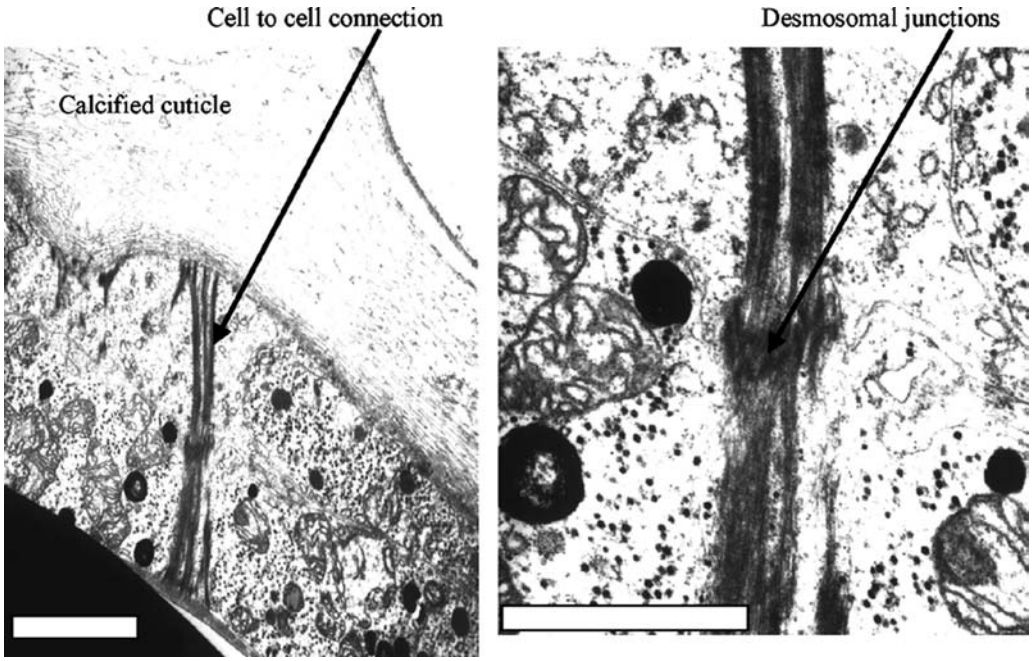

Figure 10. Cell-to-cell connections in the shell of *C. torosa* (Scale bars = 5 μm).

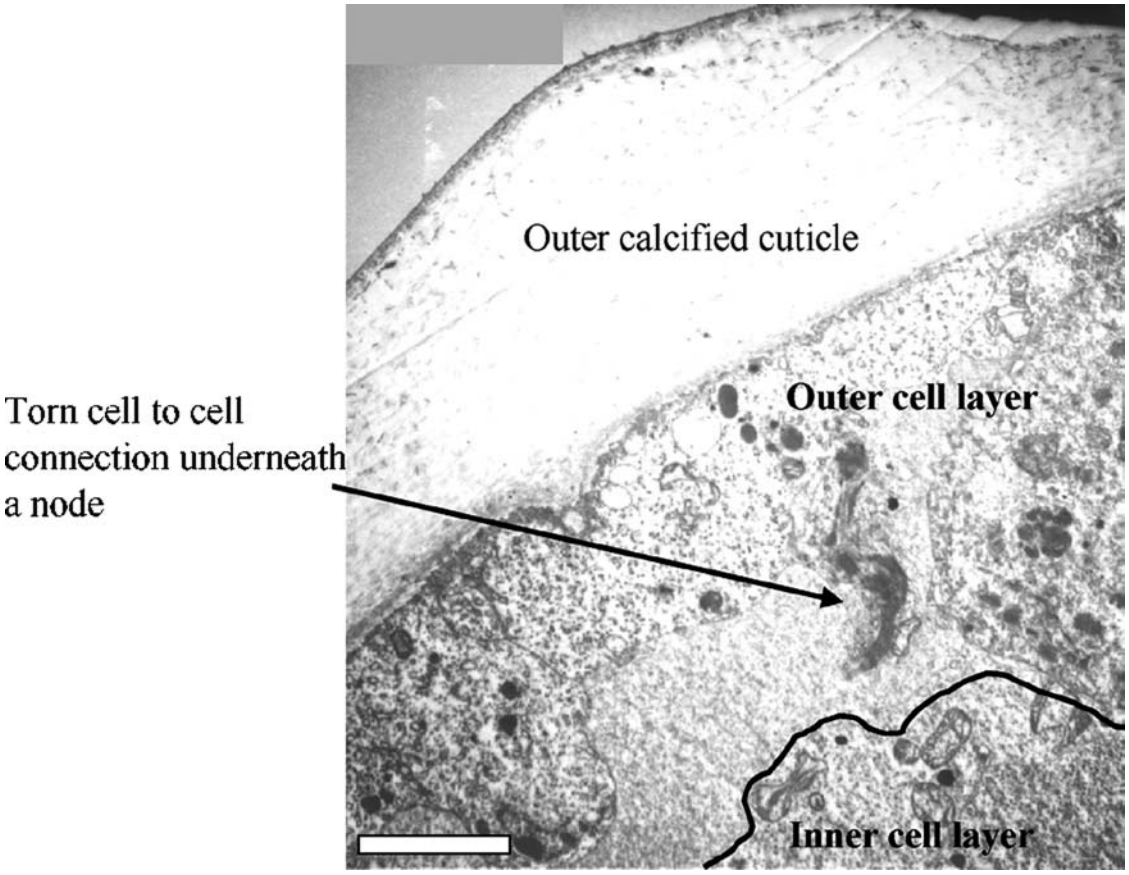

Figure 11. Torn cell-to-cell connection in *Cyprideis torosa*. Scale bar = 5 μm.

The process of noding in *C. torosa* is not genetically controlled. It is merely a pathological incident caused by the deficiency of an individual to adjust to environmental stress near its threshold of survival. It stresses the animal at the most difficult time of its life, during moulting. Here physiological shortcomings affect the ostracod more than at other times. Whether some animals in the population are better adjusted or are not affected at all by this environmental stress must still be studied. This could then lead to a systematic division of the species. The pathways of the circulatory system in the animals are certainly genetically controlled, therefore the place of the nodes on the shell is basically the same also in different members of the genus. The appearance of nodes is the response of the animal to environmental stress, namely a problem of osmolarity control.

Summary

Nodes develop on shells of *Cyprideis* in low salinity waters. They have different shapes and heights. They appear mainly at the same seven spots of the shell, which are all situated outside the isthmus which is the connection of the carapace to the body.

During moulting the cell-to-cell connections between inner and outer cell layer are destroyed causing the outer cuticle to form tubercular structures, which are later stabilized by calcification.

The noding in *C. torosa* is basically an individual problem of osmotic control. Noding is closely interlinked with the moulting process. *C. torosa* develops nodes in low saline water due to:

1. failure to adjust to the lower osmotic pressure of the surrounding water during the moulting process;

2. low amounts of calcium ions within the animal, reducing sharply the flexibility of desmosomes and muscles thereby causing some kind of a cramp and tearing of the structures involved.

These results resolve most of the problems related to noding in *Cyprideis*. It is a multifactorial system. This explains the uncertainty concerning when and to what extent nodes appear and why they are unpredictable. The fact that they are unsymmetrical on both shells is also explained.

Consequently, the use of noding as an environmental marker for low salinity and/or low calcium content is correct.

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