DIPLOMARBEIT

Titel der Diplomarbeit

Cuticle structures on different body regions of *Semibalanus balanoides* (L.) (Thoracica, Cirripedia) with special emphasis of the mature and degenerating penis

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Zusammenfassung

In dieser Arbeit wurde die Kutikula von Penis, Cirrus und Thorax des Cirripeden *Semibalanus balanoides* (L.) (Thoracica) transmissions- und rasterelektronenmikroskopisch untersucht. Ein besonderes Augenmerk galt dem reifen und degenerierenden Penis. Der reife Penis hat eine überwiegend glatte Kutikula während der äußerste Teil der Kutikula des degenerierenden Penis stark gefaltet und der innere Teil (über dem Gewebe) glatt ist. Die Kutikula des Cirrus und des Thorax ist glatt, ähnlich wie die des glatten Penis.

Die Unterschiede in Dicke, Oberflächenbeschaffenheit und Schichtung der Kutikula der verschiedenen Körperregionen wurden untersucht, verglichen und diskutiert.

Abstract

In this study the cuticle of penis, cirrus and thorax of the cirripede *Semibalanus balanoides* (L.) (Thoracica) was investigated with the Transmission and the Scanning Electron Microscope. A special emphasis was on the mature and the degenerating penis. The cuticle of the mature penis is more or less smooth whereas the exterior part of the cuticle of the degenerating penis is very much crinkled and the inner part of the cuticle (above the tissue) is smooth. The cuticle of the cirrus and the thorax is more or less smooth.

The differences in thickness, surface and layering of the cuticle of the different body regions were investigated, compared and discussed.

Key words: *Semibalanus balanoides*, cuticle, degenerating penis, mature penis, cirrus, thorax, TEM, SEM

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1. Introduction

A typical arthropod exoskeleton is a multilayered structure with two functional regions: epicuticle and procuticle. The thin dense outer epicuticle consists mainly of proteins, lipids and calcium salts. The internal procuticle is much thicker, less dense and lamellate. It is subdivided into a sclerotized outer exocuticle and an unsclerotized inner endocuticle and composed of proteins and chitin (Freeman, 1980; Koulish and Gould, 1983). Moulting of the exoskeleton is necessary for arthropods to increase in size. At the beginning of this process the endocuticle is often resorbed (mineral salts are usually withdrawn for reuse). The epicuticle and exocuticle are not digested during the moulting cycle and therefore shed at every moult. The new cuticle is excreted by the underlying epidermis. After the old cuticle is shed, the arthropod typically pumps up its body to allow the new cuticle to expand to a larger size.

In this study the cuticle of penis, cirrus and thorax of the cirripede *Semibalanus balanoides* (L.) was examined. Cirripedia are a subclass of Crustacea, being sessile or parasitic and occurring in marine or estuarine habitat. Darwin (1851; 1854) wrote the first comprehensive monograph on Cirripedia. Martin and Davis (2001) distinguished three superorders: Acrothoracica, Rhizocephala and Thoracica. The largest and most diverse group are the Thoracica with two orders, the Pedunculata and Sessilia. The latter contains the suborder Balanomorpha also known as acorn or sessile barnacles, which encompass the greatest diversity of the sessile barnacles. They are found in almost all marine habitats, from equatorial to polar regions. Balanomorpha settle on any hard substrate like rocks, stones and ship hulls and also on secondary hard bottom like seashells and other crustaceans. *Semibalanus balanoides* (L.) is a boreo-arctic cirripede occurring in the sublitoral zone.

Most barnacles are simultaneous hermaphrodites, but some of them have complemental males. Looking for a functional female, in their surrounding, they use their highly extensible, annulated penis. The penis of a functional male can be extended by turgor pressure up to four times its resting length and retracted by the longitudinal muscles (Barnes, 1992; Klepal et al., 1972). The general morphology and diversity of the cirripede penis was described by Darwin (1854) but a detailed study of the morphology and histology was made by Klepal et al. (1972). The penis, beset with four longitudinal rows of sensory setae is covered by cuticle. On the distal part of the penis the cuticle forms the inner lining of the ductus ejaculatorius. On the tip of the penis the central ejaculatory duct is surrounded by a layer of circular muscles. Between the external cuticle and the central duct is a network of haemocoelic channels in the connective tissue. Four penial nerves embedded in the connective tissue are marked externally by the four lines of setae. A number of longitudinal muscles are arranged in a ring, just beneath the epithelium.

The penis of *Semibalanus balanoides* degenerates after the short breeding season in October and November. It is separated from the body by an abscission layer which is replaced by new cuticle (Crisp and Patel, 1958) eventually. During the mouth before the next mating season the penis is regenerated to its full size and functionality.

The thorax bears six pairs of thoracopods known as cirri. A cirrus has a two-segmented pedicel from which two rami project, the anterior exopod and the posterior endopod. For feeding the cirri are extended as a fan through the opercular opening. Long setae on the cirri form a filtering or food-collecting device. The rotation of the cirral fan allows an efficient food collection by filtering the water (Ayling, 1976). Darwin (1851; 1854) recognized that the cirri are extended by haemolymph pressure while retraction is done by specific muscles, similar to the penis.

In this study the cuticle of penis, cirrus and thorax of *Semibalanus balanoides* were examined with Transmission and Scanning Electron Microscope. The differences in thickness, surface structure and layering of the cuticle were investigated and discussed. Macroscopic analyses indicate that the cuticle of the thorax is “softer” than the cuticle of the penis and the cirrus.

2. Material and Methods

Semibalanus balanoides was collected in Scotland. Penis, cirrus and thorax of some individuals were fixed in Karnovsky (4% Paraformaldehyde and 2,5% Glutaraldehyde in 0,2M Cacodylate buffer, pH 7,3 with 10% Sucrose) for 5-7 h, other individuals were fixed in 2,5 % Glutaraldehyde in 0,1M Cacodylate buffer for 2 h. The animals were postfixed in 1% Osmiumtetroxide in 0,1M Cacodylate buffer for 2 h. The samples were dehydrated in a graded series of Ethanol and infiltrated with a mixture of Acetonitrile and Resin in two steps (3:1, 1:1). *S. balanoides* was embedded in Spurr, as well as in Agar Low Viscosity Resin. Thin sections (60 nm) were cut with a Leica Ultracut S microtome and stained with Uranyl acetate (15 minutes) and Lead citrate (eight minutes). The sections were viewed in a Zeiss EM 902 Transmission Electron Microscope.

For Scanning Electron Microscopy *S. balanoides* were first fixed with Karnovsky and then with 1% Osmiumtetroxide in 0,1M Cacodylate buffer according to the TEM-protocol. The samples were dehydrated with DMP and transferred into 100% acetone 3x for 10 min. Afterwards they were conveyed into a mixture of acetone:HMDS (1:1) for 30 min each. Finally they were put into pure HMDS and while the HMDS evaporated, the samples dried over night. Then they were mounted on stubs and sputtered with gold in a Sputtercoater AGAR B7340. The samples were examined in a Philips XL 20 Scanning Electron Microscope.

With images of the Scanning and Transmission Electron Microscope the cuticle of the penis, cirrus and thorax was measured with the software program iTEM. The mean (µm) and the standard deviation were calculated. To compare the length of the inner and the outer part of the cuticle of the penis, a straight line with a defined length (mature penis: 25 µm, degenerating penis: 50 µm) was placed above the cuticle. The ends of the line were the start and end points for the length measurement of the inner and outer part of the cuticle.

For the identification of resilin, penis and cirrus were stained with toluidine blue-light green stain following Anderson and Weis-Fogh (1964). The samples were stained with a solution containing 5 mg of Toluidine blue and 5 mg of Light green per 100 ml of 50 mM Phosphate buffer (pH 7) for 48 h on a rocking platform. Afterwards the samples were transferred into pure buffer for some hours to differentiate. The specimens were analysed with a Light Microscope.

In UV-light, resilin fluoresces with a strong bluish colour with maximum intensity of about 420 nm (Anderson and Weis-Fogh, 1964). To detect autofluorescence of resilin, penis and cirrus were viewed with the Epifluorescence Microscopes Olympus BX 41 and Nikon Eclipse E800. Two filters were used, UV-1A and DAPI. The UV-1A filter cube had an excitation at wavelengths of 360-370 nm and a barrier filter at 420 nm. The DAPI filter had the excitation at 340-380 nm and the barrier filter at 435-485 nm. The fluorescence of resilin changes in its excitation spectrum when the surrounding pH is modified (Neff et al., 2000). Therefore samples were transferred to HCl (pH 2), Glycerine (pH 7) and KOH (pH 12).

3. Results

3.1. Mature penis

The investigated mature penes of *Semibalanus balanoides* are 8 to 13 mm long. The tips of the penes are 0,1 - 0,2 mm thick, the middle 0,2 - 0,3 mm and the basis 0,3 - 0,4 mm. The penes are annulated and beset with sensory setae.

3.1.1. Tip of the penis

Setae and annuli of the penis are investigated with the scanning electron microscope (Fig.1). Groups of sensory setae are arranged in four longitudinal rows. Each group bears an individual number of setae, mostly 5-7 (Fig.2). The annuli, on average 22,5 µm wide (Tab.1), are regularly arranged (Fig.2).

The cuticle is smooth (Fig.3). The epicuticle is approximately 0,22 µm thick (Tab.5). It consists of a thin and electron dense outer layer and a thicker and perpendicularly striated inner layer (Fig.3). The procuticle consists of several alternating layers of electron dense and electron lucent material and is about 1,02 µm thick (Tab.5). Exo- and endocuticle cannot be differentiated.

The ductus ejaculatorius in the tip of the penis is lined by thin cuticle beset with combs. The epicuticle consists of one electron dense layer only and is about 0,04 µm thick (Tab.5). The procuticle is composed of few layers and is on average 0,4 µm thick (Tab.5, Fig.4).

Tab.1: SEM measurements of the width of annuli from the tip of the mature penis.

Tip	Annuli	Width	Number of Measurements	10
			Mean (µm)	**22,5**
			Standard deviation	2,4

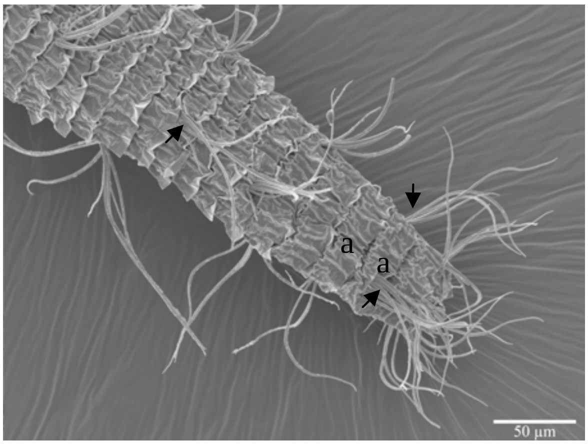


Fig.1: SEM: tip of the penis with groups of sensory setae (arrows) and annuli (a)

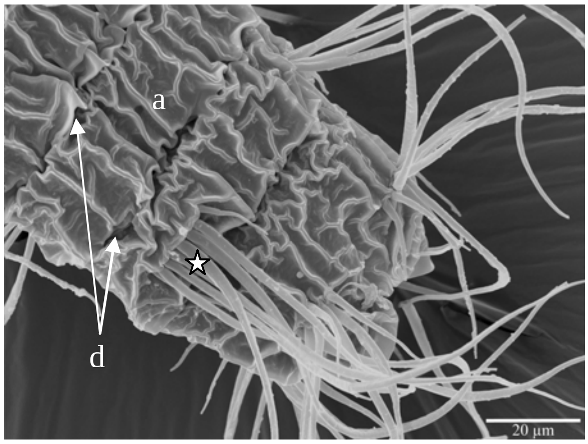


Fig.2: SEM: group of sensory setae (star) on the tip of the penis, cuticle with depressions (d) between the annuli (a)

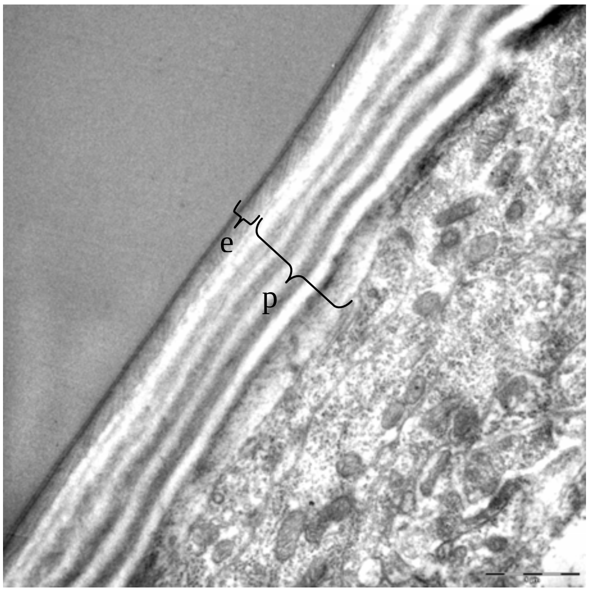


Fig.3: TEM: transverse section of the smooth cuticle on the exterior surface of the penis tip, epicuticle (e) and procuticle (p)

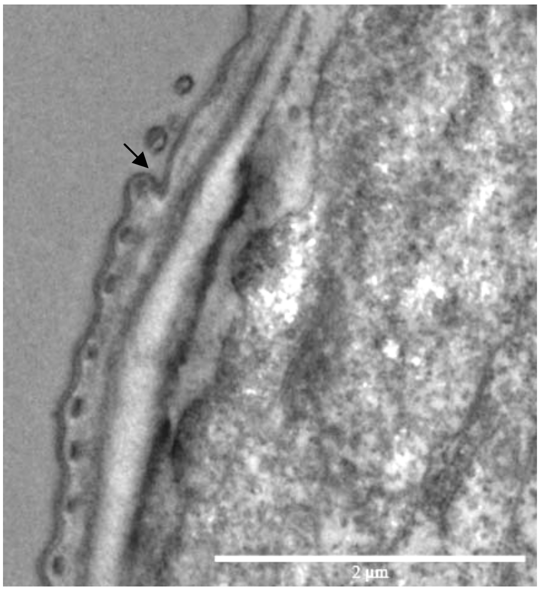


Fig.4: TEM: transverse section of penis near tip, cuticle of the inner lining of the ductus ejaculatorius with comb (arrow)

3.1.2. Middle region of the penis

In the middle of the penis only 2-4 setae form the groups. Bacteria are located in the exterior folds of the cuticle (Fig.6).

The annulations in the middle region of the penis measure about 29 µm in width (Tab.2). The cuticle of the annuli is smooth (Figs 5-7). The depressions are about 12 to 13 µm wide (Tab.2) and characterized by ridges (Figs 6-8). The height of these ridges varies between 2 and 8 µm. The depressions are on average 18,85 µm deep (Tab.2).

The epicuticle is about 0,11 µm thick (Tab.5) and consists of one electron dense layer. The procuticle is approximately 4,22 µm thick (Tab.5) and composed of several layers. The very exterior layer is the thickest. All interior layers are almost of the same thickness and lying closely together (Fig.8).

Tab.2: SEM and TEM measurements of annuli and depressions from the middle region of the mature penis.

				TEM	**SEM**
Middle region	**Annuli**	Width	Number of Measurements	7	10
			Mean (µm)	**29,13**	**29,38**
			Standard deviation	1,83	1,29
	Depression	Width	Number of Measurements	6	10
			Mean (µm)	**13,13**	**12,26**
			Standard deviation	2,18	1,25

		Depth	Number of Measurements	6	-
			Mean (µm)	**18,85**	-
			Standard deviation	3,51	-

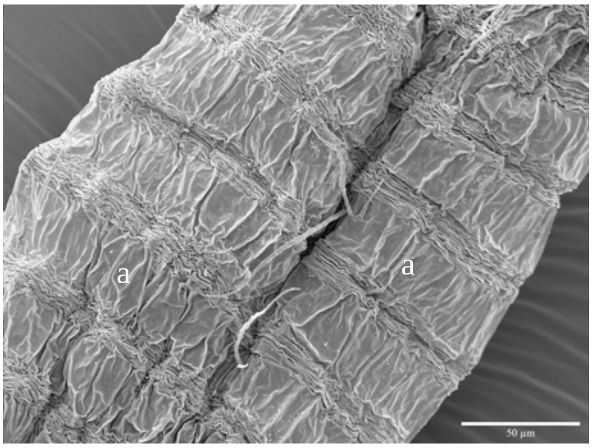


Fig.5: SEM: middle region of the penis with annuli (a) (furrow = artefact)

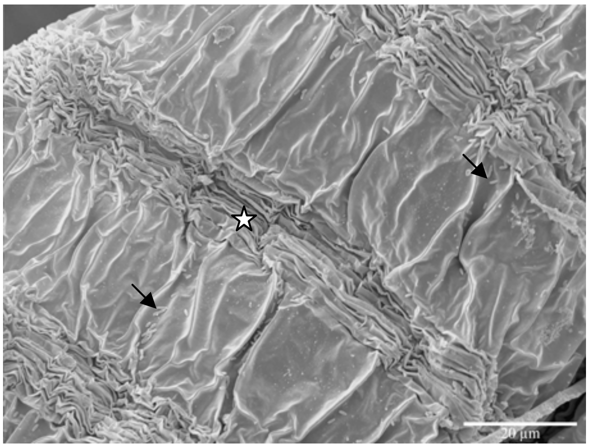


Fig.6: SEM: detail of the middle region with extended depressions (star) and bacteria on the surface (arrows)

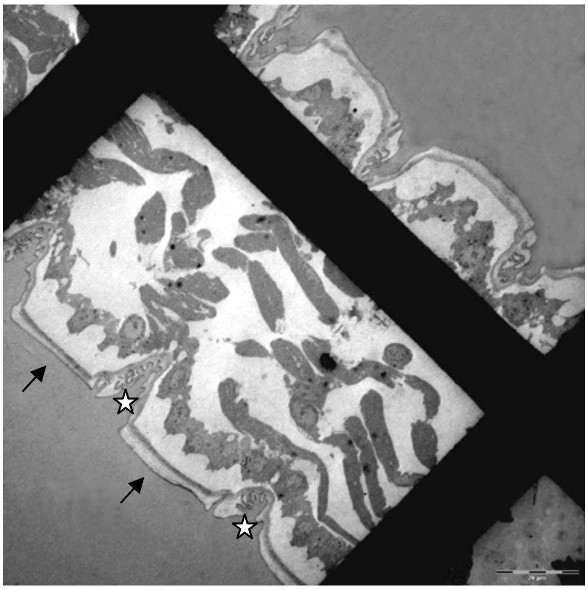


Fig.7: TEM: longitudinal section of the middle of the penis with annuli (arrows) and depressions (stars)

Fig.8: TEM: cuticle of the depression with ridges (arrows). Note wide exterior layer of procuticle.

3.1.3. Basis of the penis

The number of setae per group on the basis of the penis is smaller (1-2) than on the middle part and on the tip. The most proximal setae are shorter than those on the distal end.

The proximal annuli are not so regularly arranged as the annuli on the tip and on the middle region of the penis (Figs 9, 10). The annulations measure on average 21,69 µm in width (Tab.3).

The very exterior part of the cuticle (epicuticle and up to three outermost layers of procuticle) forms marked crinkles, whereas the remaining part is only a little bit undulated (Figs 11, 12). Consequently the outer cuticle has an increased surface area, it is almost four times larger than the remaining inner cuticle (detailed information in Tab.4). The cuticle with the crinkles is on average 6,03 µm and at maximum nearly 8 µm wide (Tab.5). The cuticle without the outer intensely wrinkled surface is about 3,64 µm wide. The epicuticle is 0,05 µm thick (Tab.5).

Tab.3: SEM measurements of annuli from the basis of the mature penis.

Basis	Annuli	Width	Number of Measurements	10
			Mean (µm)	**21,69**
			Standard deviation	3,95

Tab.4: Comparison of the length of the inner and outer part of the cuticle of the mature penis.

	Defined length (µm)	Length of the inner part of the cuticle (µm)	Length of the outer part of the cuticle (µm)	Comparison outer/inner part
Basis	25	49	186	ca. 4x longer

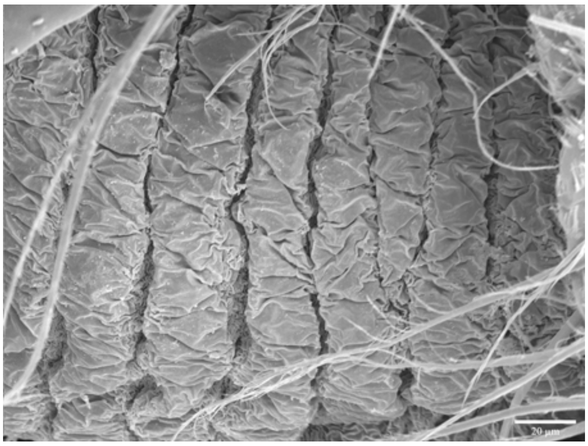


Fig.9: SEM: basis of the penis with partly irregularly arranged annuli

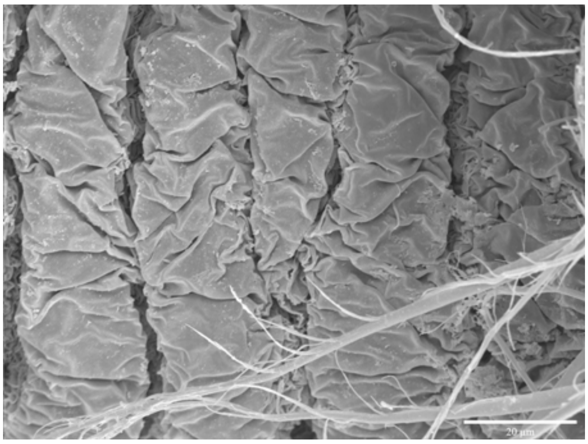
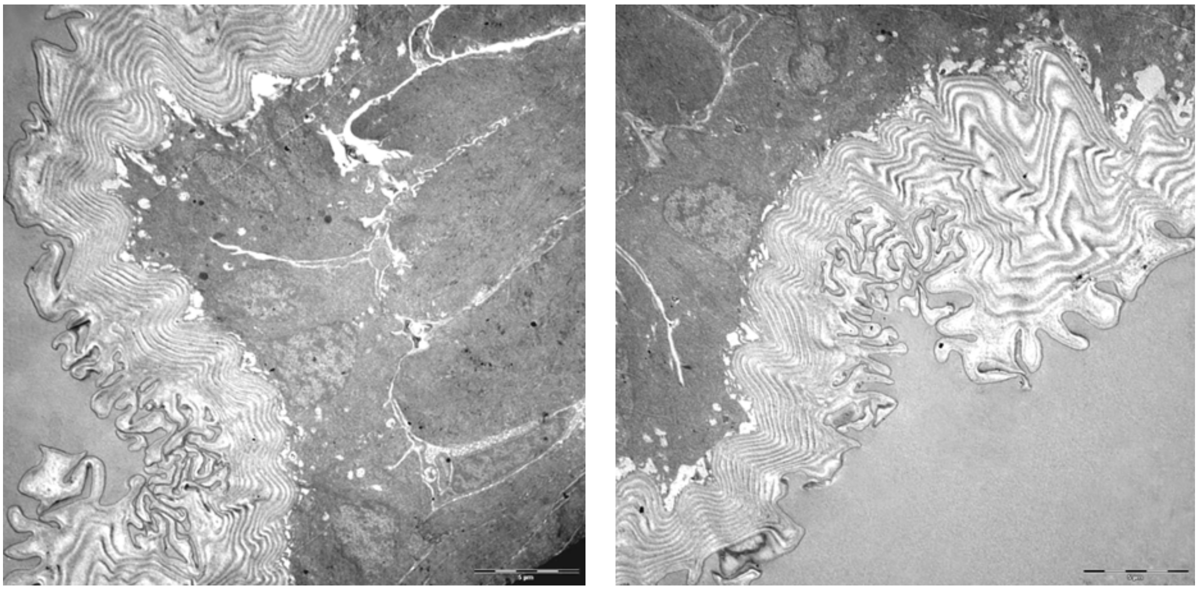


Fig.10: SEM: detail of the annuli on the basis of the penis



Figs 11, 12: TEM: transverse section of the basis, the outermost part of the cuticle forms bizzare crinkles

Tab.5: TEM measurements of the cuticle width from different regions of the mature penis.

Tip	external	Epicuticle		Number of Measurements	10
				Mean (µm)	**0,22**
				Standard deviation	0,02
		Procuticle		Number of Measurements	10
				Mean (µm)	**1,02**
				Standard deviation	0,09
	internal	Epicuticle		Number of Measurements	10
				Mean (µm)	**0,04**
				Standard deviation	0,01
		Procuticle		Number of Measurements	10
				Mean (µm)	**0,4**
				Standard deviation	0,07
Middle region		Epicuticle		Number of Measurements	10
				Mean (µm)	**0,11**
				Standard deviation	0,02
		Procuticle		Number of Measurements	10
				Mean (µm)	**4,22**
				Standard deviation	0,59
Basis		Epicuticle		Number of Measurements	10
				Mean (µm)	**0,05**
				Standard deviation	0,02
		Cuticle	with crinkles	Number of Measurements	21
				Mean (µm)	**6,03**
				Standard deviation	1,01
			without crinkles	Number of Measurements	21
				Mean (µm)	**3,64**
				Standard deviation	0,83

3.2. Degenerating penis

The degenerating penes of *Semibalanus balanoides*, which were investigated in this study, are 10 to 15 mm long. The cuticle of the annuli is beset with sensory setae and is heavily crinkled, giving the appearance of crinkled paper.

3.2.1. Tip of the penis

The scanning electron microscope shows that on the tip of the penis there are areas of the cuticle, which have large and rather smooth bulges and other areas, which have lots of little crinkles (Figs 13, 14).

In the seemingly smooth areas the cuticle overlaps itself which can be recognized under the transmission electron microscope (Fig.15). Because of these overlaps the outer part of the cuticle is about twice as long as the remaining interior part of the cuticle (detailed information in Tab.7). Bacteria are trapped by these overlaps (Fig.16).

The epicuticle is an electron dense layer about 0,5 µm wide. The procuticle is on average 7,76 µm thick (Tab.6) and consists of several alternating electron dense and lucent layers (Figs 15, 16). Exo- and endocuticle cannot be distinguished.

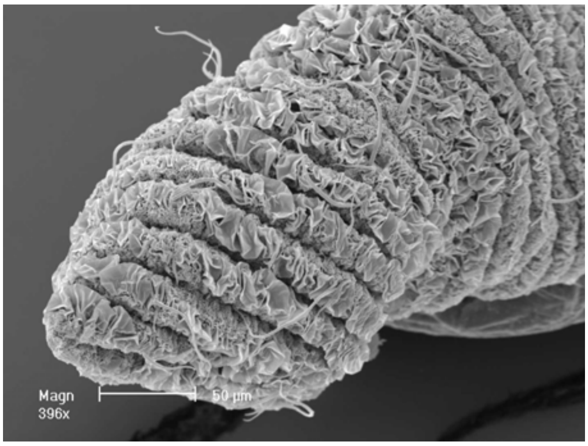


Fig.13: SEM: wrinkled cuticle on the tip of the penis

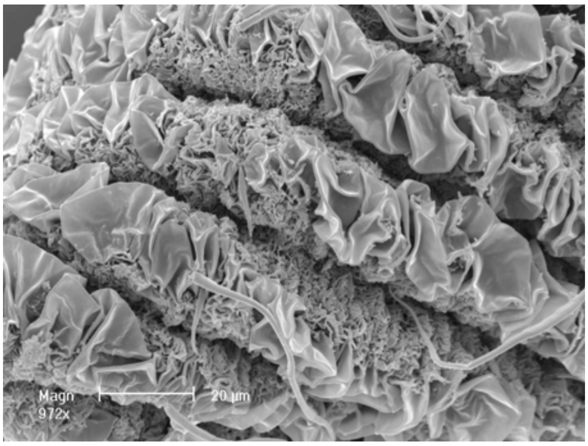


Fig.14: SEM: detail of the wrinkled surface of the cuticle

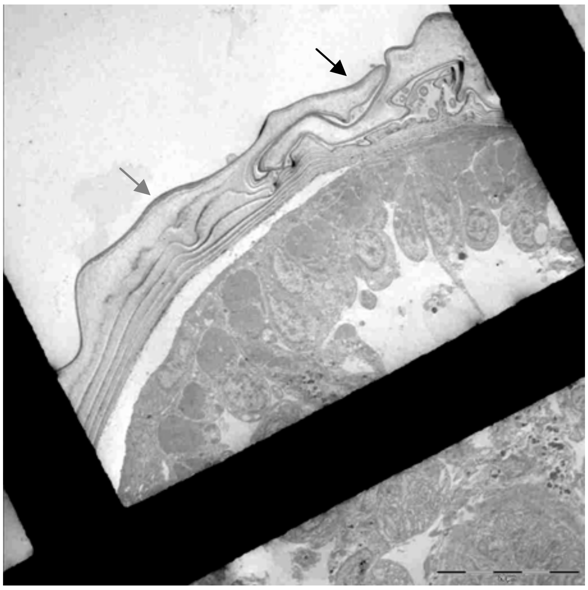


Fig.15: TEM: transverse section, smooth cuticle (grey arrow) and overlapping cuticle (black arrow)

Fig.16: TEM: bacteria are located under the overlapping cuticle (arrows)

3.2.2. Middle part of the penis

Pictures of the middle part of the penis, which were made with the SEM, look almost the same as the pictures of the tip of the penis. The cuticle is crinkled, but there are also areas with rather smooth bulges (Figs 17, 18).

TEM images show that some outermost parts of the cuticle overlap and the underlying cuticle is crinkled whereas the inner part, adjoining the epithelium, is smooth (Figs 19, 20). In cross section the surface of the exterior part of the cuticle is about six times longer than the inner part (detailed information in Tab.7). Between the crinkles as well as over and under the over-laps bacteria are located (Fig.20).

The cuticle without any crinkles and overlaps is on average 9,26 µm wide, with them it is 13,14 µm thick. The electron dense epicuticle measures approximately 0,26 µm (Tab.6). The procuticle is composed of several alternating electron dense and electron lucent layers (Figs 19, 20).

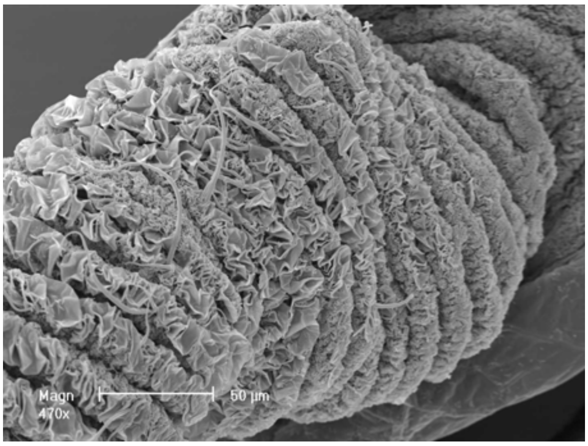


Fig.17: SEM: middle part of the penis with a crinkled surface

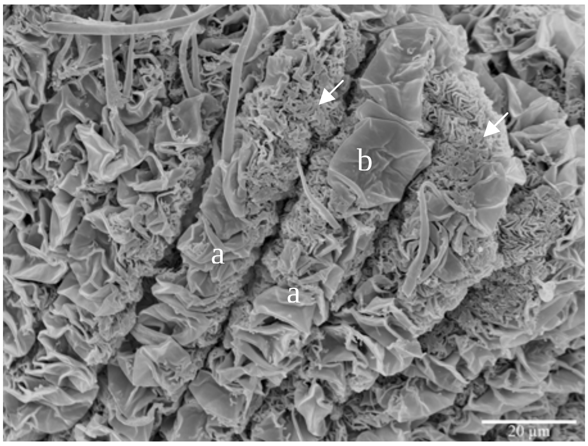


Fig.18: SEM: detail of annuli (a) with crinkles (arrows) and smooth bulges (b)

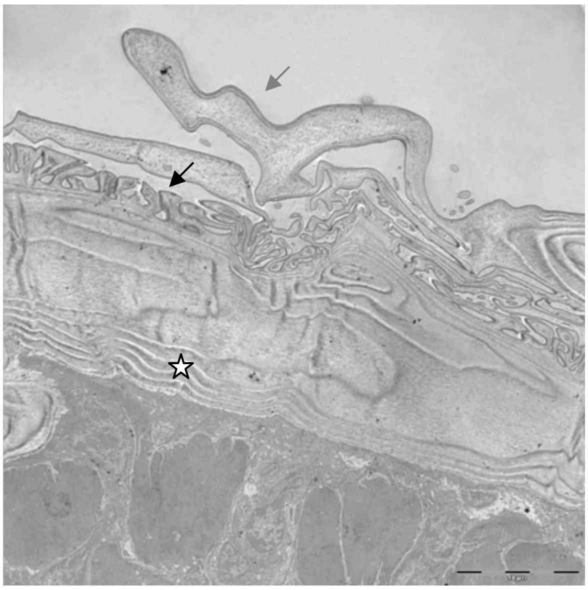


Fig.19: TEM: transverse section, the very exterior part of the cuticle overlaps (grey arrow) the crinkled exterior part (black arrow), the interior part is smooth (star)

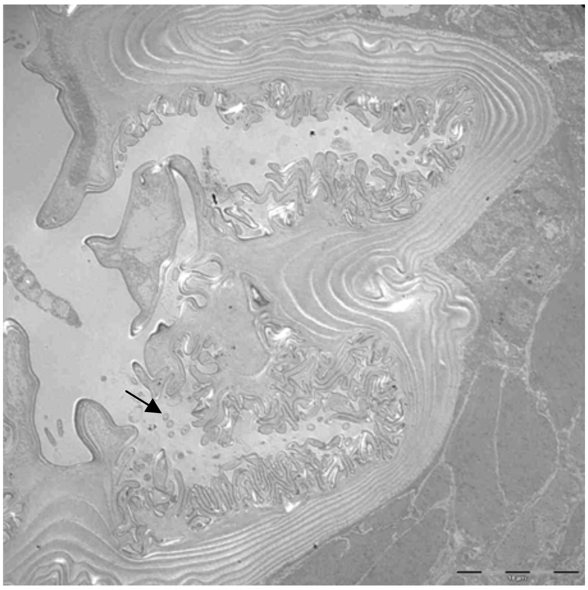


Fig.20: TEM: crinkled outer surface of the middle part of the penis, where bacteria are located (arrow)

3.2.3. Basis of the penis

SEM and TEM images show that the cuticle on the basis of the penis is crinkled most (Figs 21-26). The narrow annuli are irregularly arranged and close together (Figs 21, 22). As in the other portions of the penis the inner part of the procuticle is smooth, whereas the epicuticle and the outer part of the procuticle are crinkled (Figs 23-25).

Out of the smooth cuticle crinkles arise, which look like V and W (Figs 23, 24). They are almost upright to the interior smooth part of the cuticle. The transverse sections (Figs 23, 24) show that these crinkles are stacked upon each other. The outermost parts of the cuticle overlaps (Figs 23-25) and bacteria are situated between the cavities of the crinkles and under the overlaps (Fig.24). Because of the numerous crinkles and overlaps the exterior part of the cuticle is seven times longer than the interior part (detailed information in Tab.7). The cuticle without the crinkles is on average 7,29 µm, with them it is 13,17 µm wide. The epicuticle measures about 0,13 µm (Tab.6).

Fig.26 shows that there are samples with three zones of cuticle. The inner part of the cuticle is undulated, the middle is smooth and the outer is crinkled. The undulated region measures on average 3,95 µm and the smooth part 6,5 µm in width (Tab.6). The crinkles differ greatly in size. The smallest are 1,5 µm, the biggest 11 µm wide.

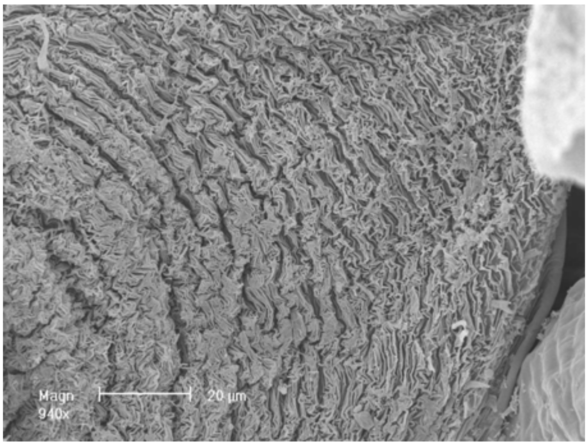


Fig.21: SEM: basis of the penis with irregular annuli, which are close together

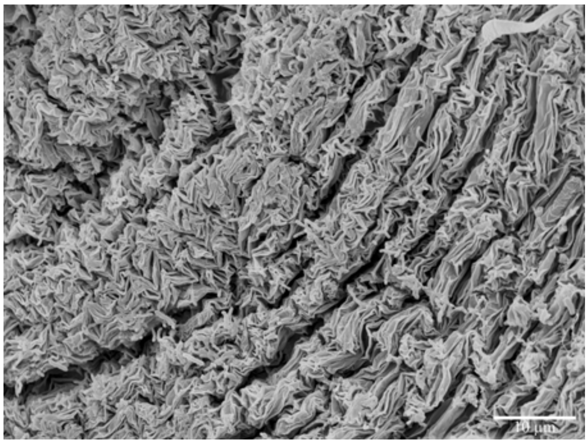
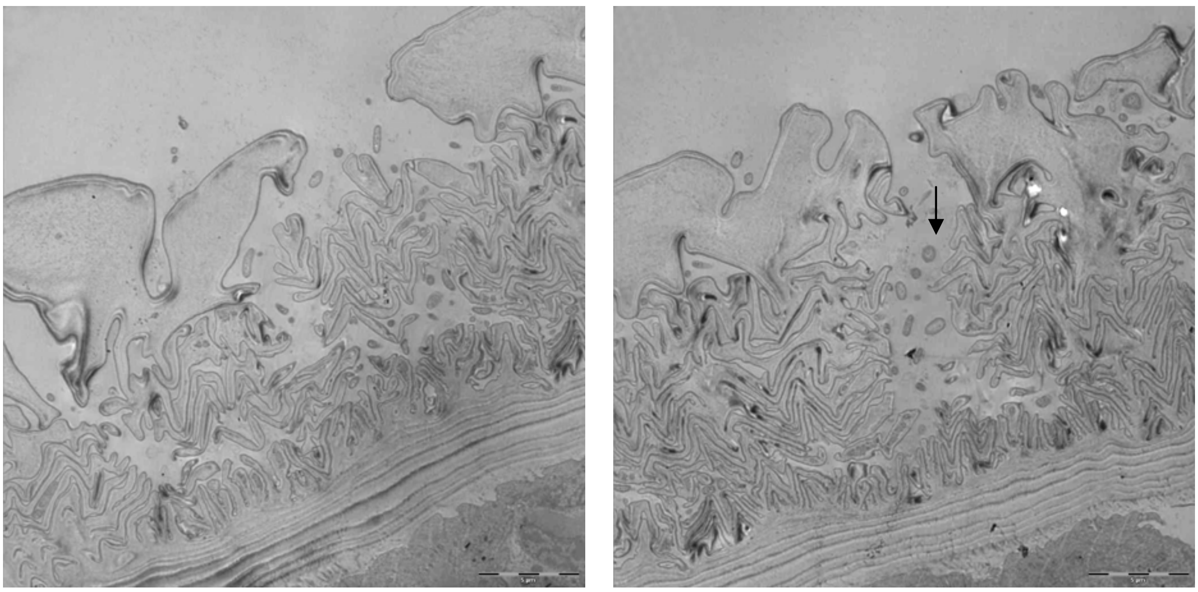


Fig.22: SEM: detail of the highly crinkled surface, annuli are difficult to separate



Figs 23, 24: TEM: transverse section, out of the smooth inner part of the cuticle crinkles arise (look like V and W), bacteria are situated between the cavities of the crinkles (arrow)

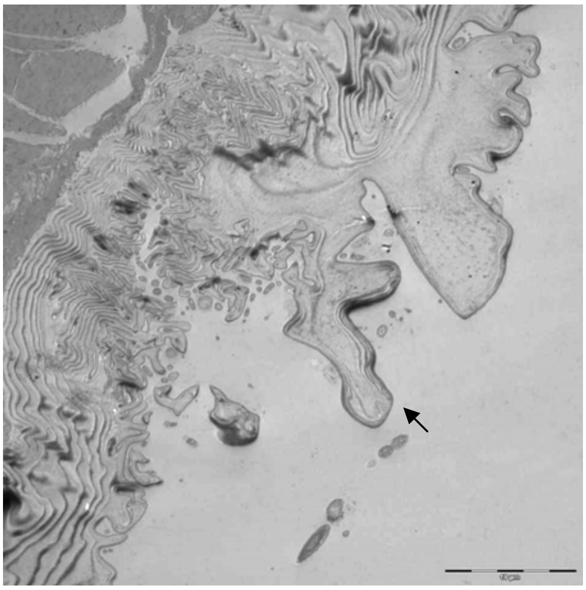


Fig.25: TEM: the outer part of the cuticle overlaps (arrow) the remaining inner part

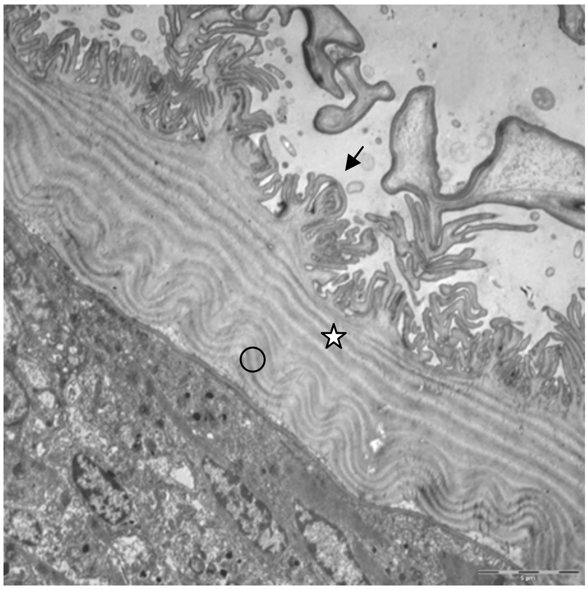


Fig.26: TEM: three zones of cuticle: inner undulated (circle), middle straight (star), outer crinkled (arrow)

Tab.6: TEM measurements of the cuticle width from different regions of the degenerating penis.

Tip	Epicuticle		Number of Measurements	10
			Mean (µm)	**0,51**
			Standard deviation	0,2
	Procuticle		Number of Measurements	10
			Mean (µm)	**7,76**
			Standard deviation	2,32
Middle region	Epicuticle		Number of Measurements	10
			Mean (µm)	**0,26**
			Standard deviation	0,04
	Cuticle	with crinkles	Number of Measurements	10
			Mean (µm)	**13,14**
			Standard deviation	4,48
		without crinkles	Number of Measurements	10
			Mean (µm)	**9,26**
			Standard deviation	4,1
Basis	Epicuticle		Number of Measurements	10
			Mean (µm)	**0,13**
			Standard deviation	0,05
	Cuticle	with crinkles	Number of Measurements	21
			Mean (µm)	**13,17**
			Standard deviation	6,54
		without crinkles	Number of Measurements	20
			Mean (µm)	**7,29**
			Standard deviation	4,06
	Samples with three zones of cuticle	undulated	Number of Measurements	20
			Mean (µm)	**3,95**
			Standard deviation	1,69
		straight	Number of Measurements	20
			Mean (µm)	**6,5**
			Standard deviation	2,57
		crinkled	Number of Measurements	20
			Mean (µm)	**4,61**
			Standard deviation	3,09

Tab.7: Comparison of the length of the inner and outer part of the cuticle of the degenerating penis.

	Defined length (µm)	Length of the inner part of the cuticle (µm)	Length of the outer part of the cuticle (µm)	Comparison outer/inner part
Tip	50	53	119	ca. 2x longer
Middle region	50	51	299	ca. 6x longer
Basis	50	53	376	ca. 7x longer

3.3. Cirrus

The cirri of mature individuals cannot be distinguished from those in the anecdysial phase which have a degenerating penis. Both look the same. The cuticle is plane (Fig.31) with combs, scales, and sensory setae (Figs 27-30) on the surface. Scales are found on the basis of the cirri arranged on prominent pads (Fig.28, also Fig.33). The scales on the pads are surrounded by combs (Fig.28). At the periphery of the pads some combs transform into scales (Fig.28).

The cirri are articulated. Each “segment” has combs and sensory setae on its distal end. Large combs overlap the joint to the following “segment” (Fig.27). Bacteria are located on the surface of the cuticle (Figs 27, 29).

The thin and electron dense epicuticle is about 0,07 µm thick (Tab.8). In the procuticle exo- and endocuticle can be distinguished (Fig.31). Both consist of several layers. The exocuticle, 1,22 µm wide, is electron dense in contrast to the endocuticle (1,28 µm wide, Tab.8).

Between the cirri the outer part of the cuticle has ridges and depressions, whereas the inner part of the cuticle is plane (Fig.32).

Tab.8: TEM measurements of the cuticle width of the cirrus.

Epicuticle	Number of Measurements	10
	Mean (µm)	**0,07**
	Standard deviation	0,04
Exocuticle	Number of Measurements	12
	Mean (µm)	**1,22**
	Standard deviation	0,17
Endocuticle	Number of Measurements	12
	Mean (µm)	**1,28**
	Standard deviation	0,22

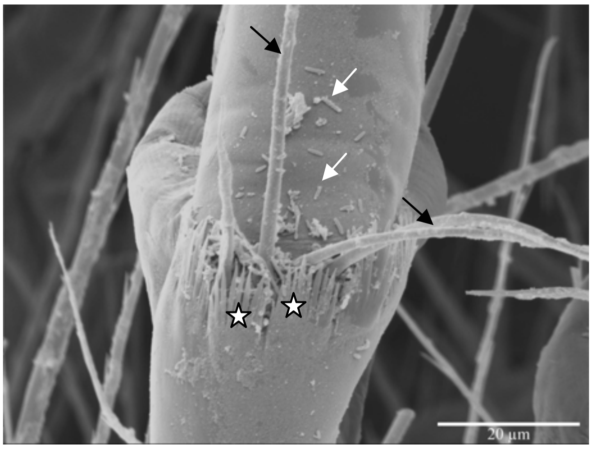


Fig.27: SEM: dorsal view of the distal end of one "segment" with combs (stars) and sensory setae (black arrows), bacteria on the surface (white arrows)

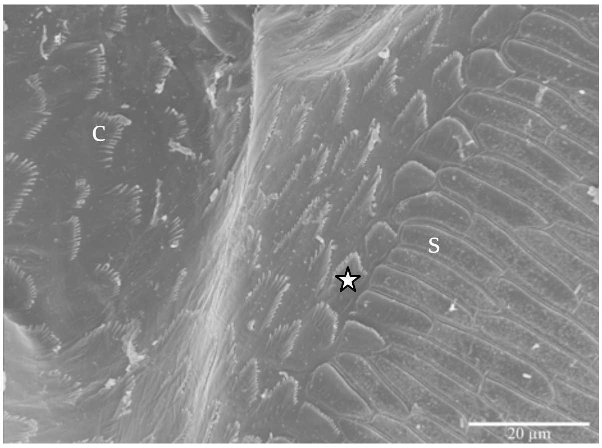


Fig.28: SEM: cuticle of the cirrus with scales (s) on a pad surrounded by combs (c), in between transformation of combs into scales (star)

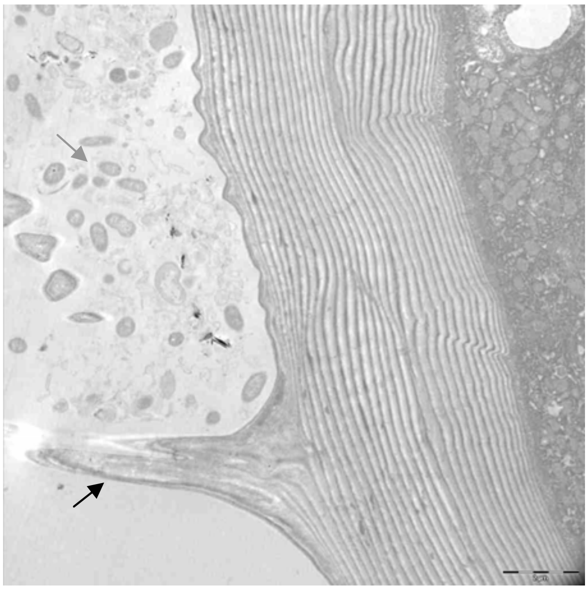


Fig.29: TEM: transverse section, cuticle with sensory seta (black arrow) and bacteria (grey arrow)

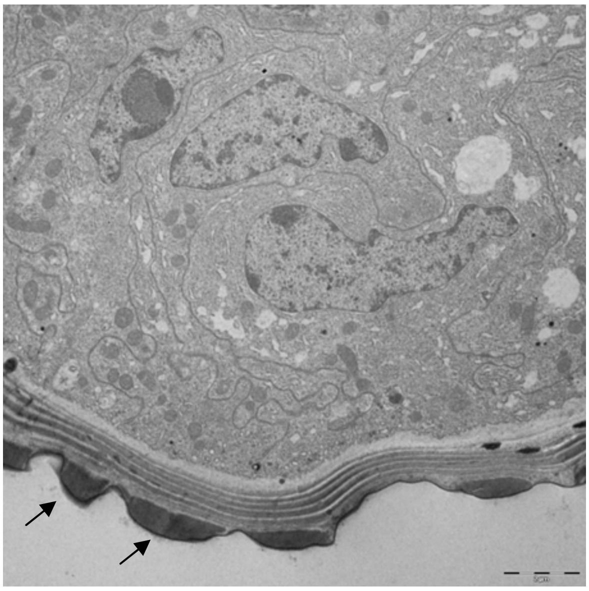


Fig.30: TEM: transverse section of the cuticle, which has scales presumably on a pad (arrows)

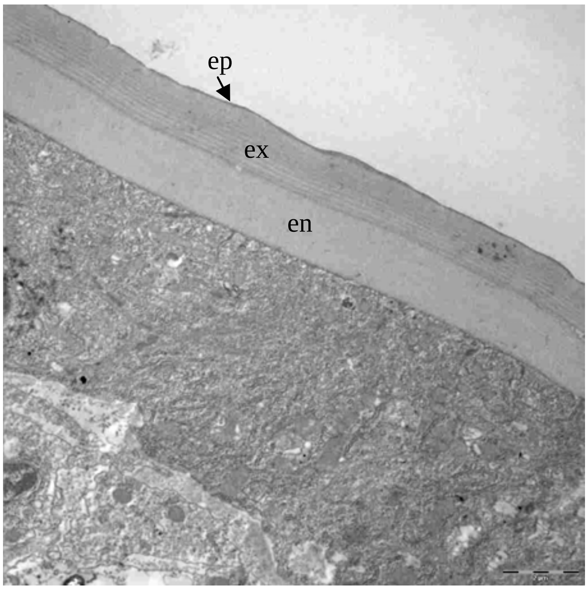


Fig.31: TEM: plane cirral cuticle differentiated into epi-(ep), exo- (ex) and endocuticle (en)

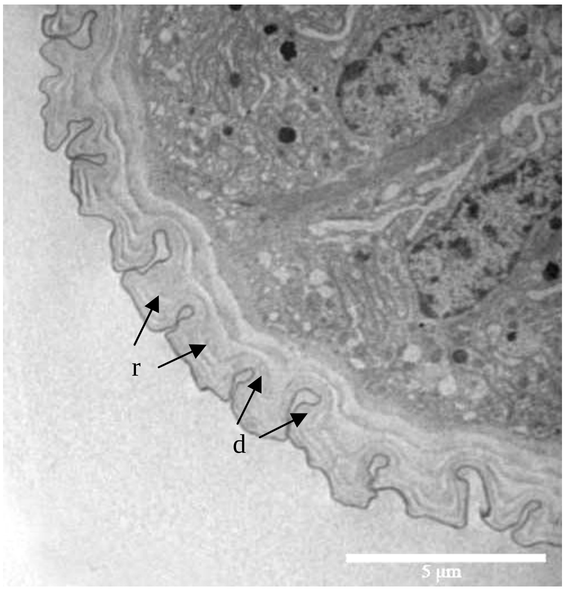


Fig.32: TEM: cuticle between cirri with ridges (r) and depressions (d)

3.4. Thorax

The thorax of mature and individuals in the anecdysial phase looks the same. In the thin cuticle a procuticle, on average 0,51 µm thick, and an epicuticle, which measures about 0,04 µm, can be distinguished (Tab.9). The procuticle consists of alternating electron dense and electron lucent layers (Figs 35, 36). The cuticle of the thorax is not as smooth as the cuticle of the cirrus. The exterior part is slightly undulated whereas the interior part is smooth (Figs 35, 36). Also on the thorax pads exist elevating from the surrounding area (Fig.33). Scales are present on these pads and combs (most of them transform into scales) encompass the pad (Fig.34).

Tab.9: TEM measurements of the cuticle width of the thorax.

Epicuticle	Number of Measurements	10
	Mean (µm)	**0,04**
	Standard deviation	0,02
Procuticle	Number of Measurements	15
	Mean (µm)	**0,51**
	Standard deviation	0,1

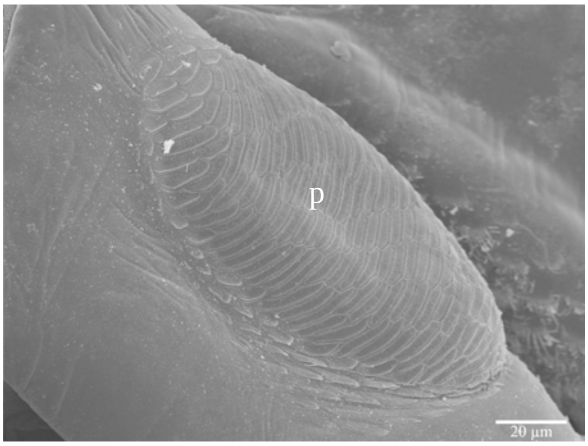


Fig.33: SEM: pad (p), which is more prominent than the surrounding area

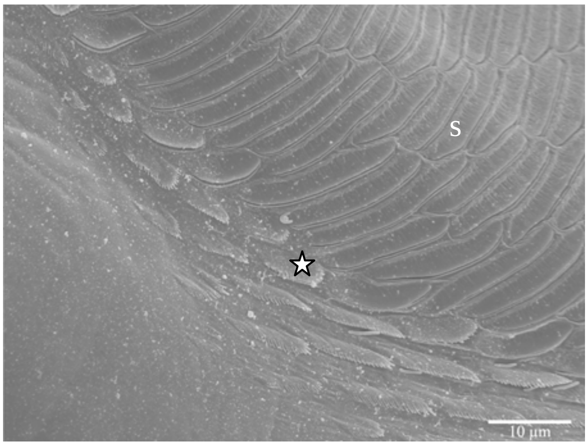


Fig.34: SEM: periphery of the pad, combs transform into scales (star), on the pad scales are located (s)

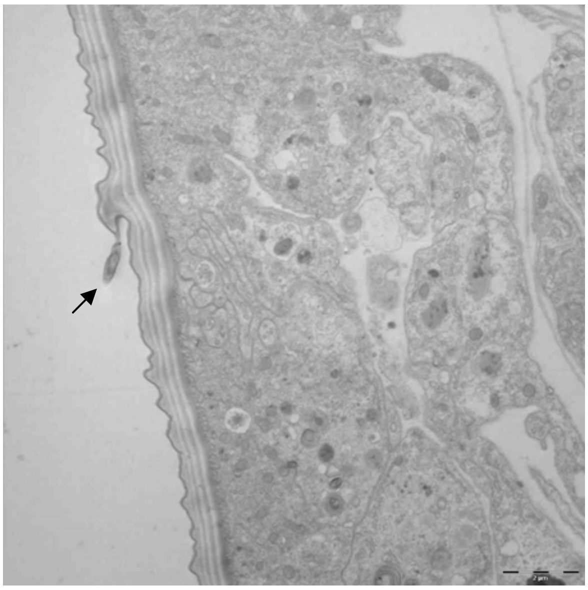


Fig.35: TEM: transverse section of the thorax cuticle, exterior part slightly undulated with comb (arrow)

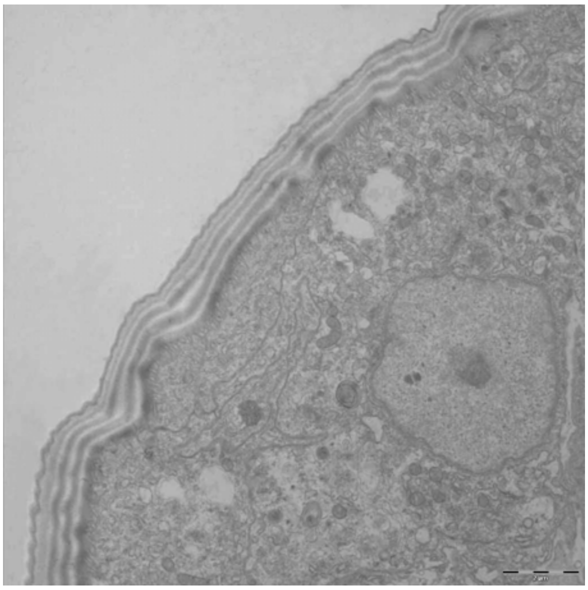


Fig.36: TEM: smooth cuticle with exterior part slightly undulated

3.5. Cuticle - summary

The thickness of the **epicuticle** increases from the proximal to the distal end of the mature as well as of the degenerating penis. The epicuticle of the degenerating penis is almost more than twice as thick as the epicuticle of the mature penis (Tab.10). The epicuticle of the basis of the mature penis, the cirrus and the thorax is very thin and measures almost the same in all three regions (about 0,05 µm, Tab.10).

Tab.10: TEM measurements of the epicuticle width from the different body regions in comparison.

	Epicuticle (mean of ten measurements in µm)		
	Tip (external)	Middle	Basis
Mature penis	0,22	0,11	0,05
Degenerating penis	0,51	0,26	0,13
Cirrus	0,07		
Thorax	0,04		

The **procuticle** of the tip of the mature and of the degenerating penis is thinner than on the other regions of the penis (Tab.11). The thickest procuticle is on the basis of the penis with crinkles on the mature and on the degenerating penis, but the basis without the crinkles is thinner than the procuticle of the middle region of the penis. The procuticle of the cirrus is thicker than on the tip of the mature penis, but thinner than on the remaining part of the mature penis. The procuticle of the thorax is the thinnest in comparison with the other body regions (Tab.11).

Tab.11: TEM measurements of the procuticle width from the different body regions in comparison. In bracket is the number of measurements.

	Procuticle (mean µm)				
	Tip (external)	Middle		Basis	
		without crinkles	with crinkles	without crinkles	with crinkles
Mature penis	1,02 (10)	4,22(10)	-	3,59 (21)	5,98 (21)
Degenerating penis	7,76 (10)	9,0 (10)	12,88 (10)	7,15 (20)	13,03 (21)
Cirrus	2,5 (12)				
Thorax	0,51 (15)				

3.6. Resilin

3.6.1. Toluidin blue-light green stain

After staining the penis with toluidine blue-light green and differentiating with buffer for four hours, the colour vanished from the tip. The middle portion is stained light blue, the basis dark blue (Fig.37). After six hours in buffer the colour disappeared completely (Fig.38).

Fig.37: Penis after four hours in buffer, blue colour at basis and middle, none at tip (x4)

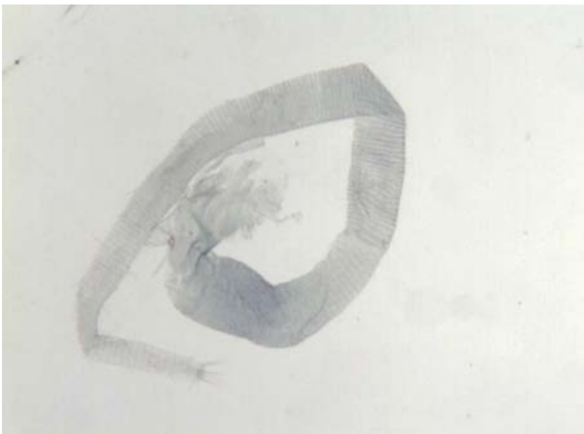


Fig.38: Penis after six hours in buffer, colour vanished (x4)

After four hours differentiating in buffer the blue colour vanished from the cirrus, only the joints of the “segments” are stained blue (Fig.39). After six hours no blue colouration is noticeable (Fig.40).

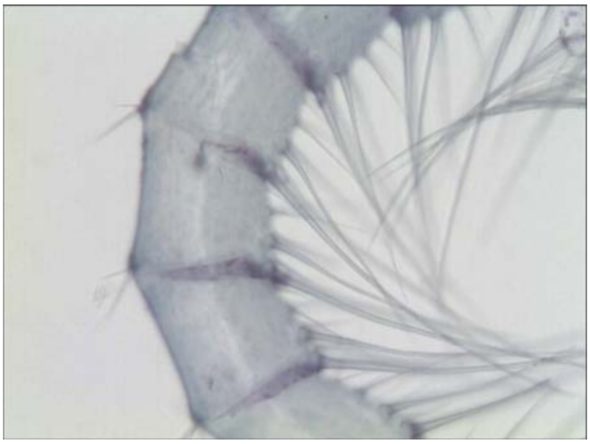


Fig.39: Cirrus after four hours in buffer, blue colour detected at the joints of the “segments” (x10)

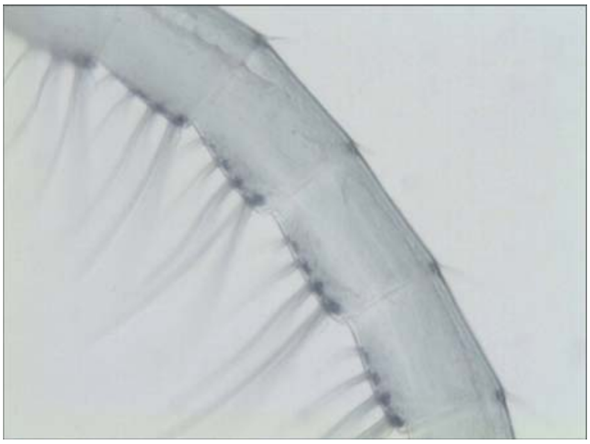


Fig.40: Cirrus after six hours in buffer, no blue colour noticed (x20)

3.6.2. pH dependence of UV fluorescence

Examination of the cuticle from the middle of the penis with the epifluorescence microscope (UV-1A and DAPI filters) shows that the entire cuticle fluoresced blue in Glycerine as well as in HCl and in KOH (Figs 41-46). In Glycerine (pH neutral) the penis fluoresces almost turquoise with the UV-1A filter (Fig.41) and has a dark blue coloration with the DAPI filter (Fig.42). With the UV-1A filter (Fig.43) the penis in HCl appears brighter than with the DAPI filter (Fig.44). The penis in the alkaline solution (KOH) has a lucid bluish colour with the DAPI filter (Fig.46), with the UV-1A filter the penis appears light blue (Fig.45).

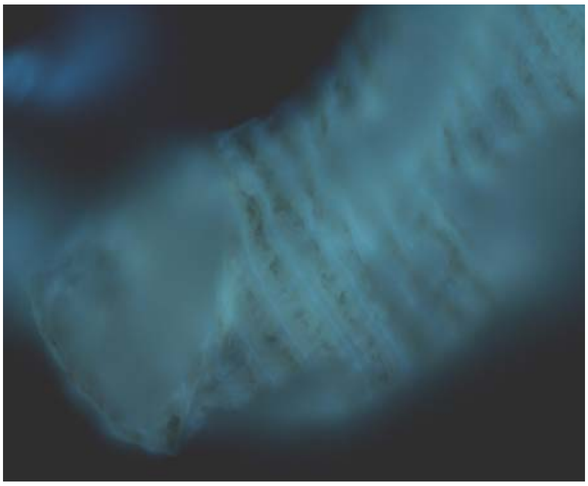


Fig.41: Middle of the penis in Glycerine shows an almost turquoise colour with the UV-1A filter (x40)

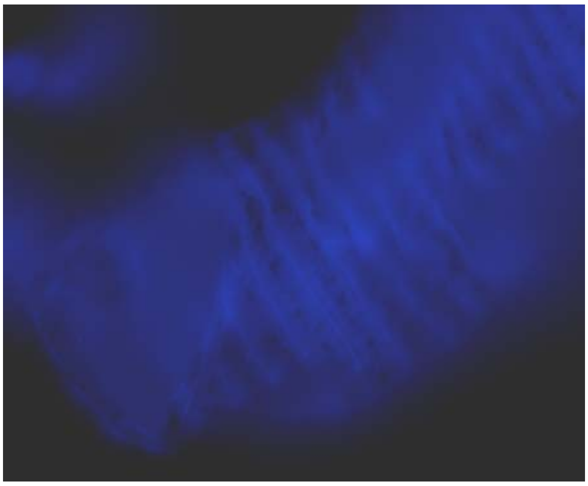


Fig.42: Middle of the penis in Glycerine has a dark blue dyeing with the DAPI filter (x40)

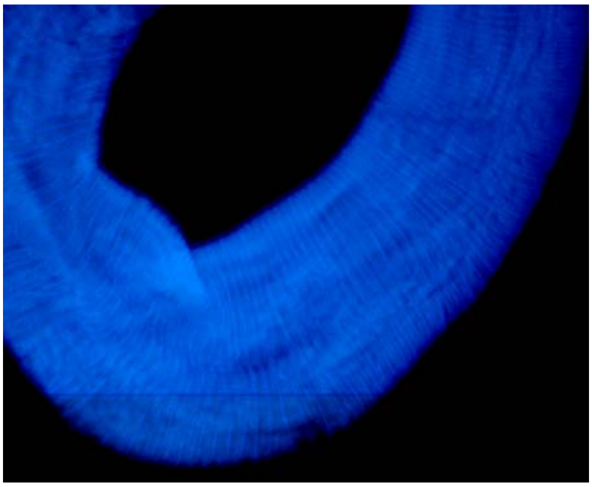


Fig.43: Middle of the penis in HCl appears light blue with the UV-1A filter (x10)

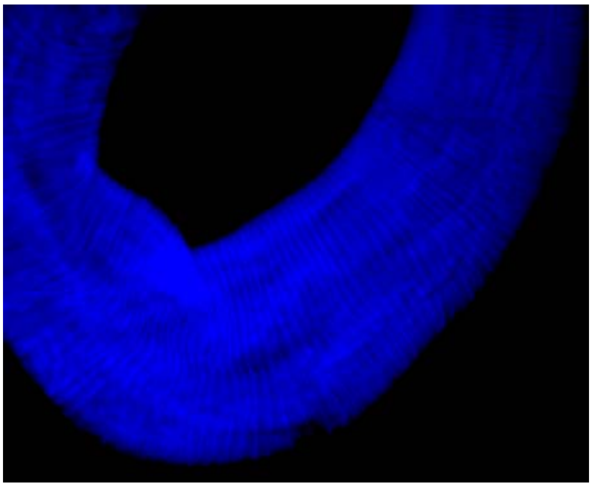


Fig.44: Middle of the penis in HCl is strong blue coloured with the DAPI filter (x10)

Fig.45: Middle of the penis in KOH is light blue coloured with the UV-1A filter (x10)

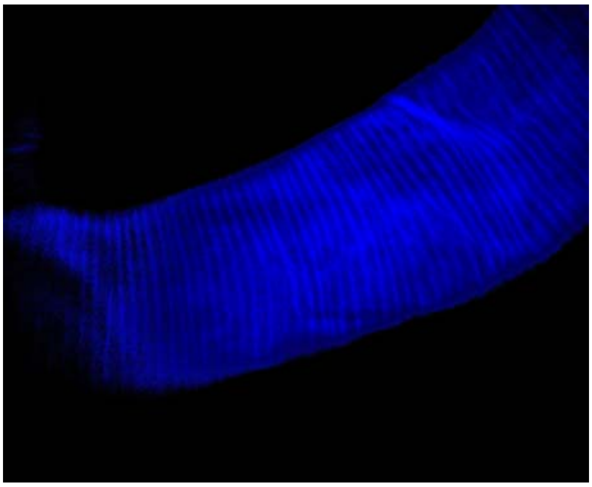


Fig.46: Middle of the penis in KOH has a lucid bluish colour with the DAPI filter (x10)

4. Discussion

4.1. Mature penis

The penis is an annulated and highly extensible organ, which can be extended up to four times its resting length (Klepal et al., 1972). The depressions are important for the increase in length under turgor pressure; they allow extension of the penis. Klepal et al. (1972) interpret the ridges in the depressions as causing a certain rigidity in the extended penis during copulation.

The proximal annulations of the penis are narrower and more irregularly arranged than the distal ones and the cuticle of the annuli has more crinkles proximally. Also the number of the ridges increases in the more proximal depressions (Klepal et al., 1972). The maximum deflexion is achieved at the basis of the penis. The cuticle there should be stable and therefore the crinkles and ridges might be necessary in such a great number.

The outer cuticle of the mature penis is more or less plane. On the distal part of the penis the cuticle forms the inner lining of the ductus ejaculatorius (Walley, 1965). This cuticle is ornamented with combs, which can be found on other parts of the body but which are absent on the outer cuticle of the penis (Klepal et al., 1972).

The position, arrangement and number of setae on the penis are described accurately by Klepal et al. (1972), which concur with my results.

4.2. Degenerating penis

After copulatory activity has ceased, the penis degenerates and is lost. Klepal et al. (1975) describe the variability in the loss of the penis and its degeneration. Not only *Semibalanus balanoides* and other Cirripedia Thoracica (e.g. *Balanus balanus, Chthamalus montagui, C. stellatus, Verruca stroemia;* Barnes, 1992) lose the penis, there are also other invertebrates which shed the penis after the breeding season. *Nassarius obsoletus* (Nassariidae, Mollusca) resorbs the penis at the end of the breeding season and regenerates it almost immediately (Jenner and Chamberlain, 1955). In *Littorina littorea* (Littorinidae, Mollusca) the penis is shed at the end of breeding. Shortly after shedding, a new penis is grown (Grahame, 1969).

In almost every major group of Crustacea the moulting cycle is often interrupted after copulation, so that eggs are not lost by shedding of the cuticle to which they are attached (Crisp and Patel, 1958). An exception are Cirripedia, which have the egg masses lying freely in the mantle cavity during incubation. As a result they do not necessarily lose them when they moult (Crisp, 1956). But Crisp and Patel (1958) investigated six species of sessile barnacles and they found that the intermoult period is prolonged when the barnacle is carrying embryos. Normally ecdysis does not occur until the nauplii have hatched and escaped from the mantle cavity.

This might be important for my first hypothesis, which can explain the crinkled cuticle of the degenerating penis. Furthermore the repair cuticle, which is produced by Crustacea after injury, plays the second considerable role in my hypothesis. A repair cuticle produces no epicuticle across the wound (Halcrow and Smith, 1986). The absence of an epicuticle might be a general property of the repair cuticle produced by arthropods especially Crustacea (Halcrow, 1988). Now to my hypothesis: It is conceivable that the penis of *Semibalanus balanoides* is injured in trying to withdraw from the mantle cavity through the closing operculum of the functional female. Due to the following wound healing, a new smooth repair cuticle is laid down. This new cuticle is only composed of procuticle, no epicuticle is formed. The old cuticle, which is not shed after the breeding season, remains folded and crinkled.

The second hypothesis to explain the crinkles suggests that the elongation of the penis during searching movements and copulation (to four times of its resting length) may cause an over-expansion of the cuticle. When the penis retracts the inner procuticle contracts due to its elasticity. The outer procuticle and epicuticle remain expanded. This results in the inner surface of the procuticle being smooth and almost straight, the outer procuticle and epicuticle being highly crinkled. The basic assumption for this hypothesis are different elastic properties of inner and outer procuticle. To check that histochemical tests for resilin, a rubberlike protein, were applied. Identifying resilin with the toluidine blue-light green stain (Anderson and Weis-Fogh, 1964) lead to no clear result. The blue colour in the cuticle of my samples vanished after six hours in buffer, unlike the tissues from Neff et al. (2000), who viewed the tissues until they were six hours in buffer.

Also the pH dependence of UV fluorescence test after Neff et al. (2000) yielded in no clear results. My samples fluoresced blue in Glycerine as well as in HCl and in KOH. Neff et al. (2000) state that the intense fluorescence is at neutral pH, at pH 2 the fluorescence is completely suppressed and reversibly returned at pH 12. Anderson and Weis-Fogh (1964) say that the intensity increases in alkaline media. In contrast my specimens show the lowest fluorescence in KOH (UV-1A filter).

A reason for my results differing greatly from those by Neff et al. (2000) might be the use of different samples. In this study whole penes and cirri were used whilst Neff et al. (2000) worked with soft tissues. Further investigations with histological sections might elucidate the absence or presence of resilin in the cuticle of penis and cirri.

4.3. Cirrus

Semibalanus balanoides lives on boreal rocky shores in shallow water. This species performs the following cirral activities: pumping beat and prolonged cirral extension in fast water currents, and normal and fast beat in still and slowly moving water (Crisp and Southward, 1961; Crisp and Maclean, 1990; Trager et al., 1990).

The cirri are extended forming a fan. Its rotation allows efficient food collection over a wide range of water. The entanglement of the several cirri with each other is most unlikely. A reason for this might be the cuticular ridges between the bases of the cirri, which may cause a certain stiffness and thus separation of the individual cirri in the fan.

Not only sensory setae but also non-sensory cuticular structures are found on the cirri. Mainly scales and combs can be identified, which are defined by Klepal and Kastner (1980). The assembly of scales, which are located at the lateral side of the cirrus basis, are more prominent than the surrounding area. Klepal and Gruber (1998) describe these areas as pads, which are surrounded by combs. The pads are interpreted by these authors as grooming devices that keep the body surface free of microbial organisms, debris, sediment and detritus.

4.4. Thorax

The thorax is covered by a thin cuticle, because in the shell it is protected and safe from predators.

Pads are also on the thorax. The entirety of the pads on the thorax and on the cirri may act after Klepal and Gruber (1998) as a sealing against particles during filter feeding. These pads ensure an almost complete protection of the body surface in the shell.

4.5. Cuticle

From the distal to the proximal end of the penis the thickness of the procuticle increases just as the number of the crinkles. Consequently the cuticle on the basis may give a certain rigidity. Unlike the procuticle, the epicuticle width increases from the proximal to the distal end of the penis.

The cuticle of the annuli of the mature penis, the cirrus and the thorax is smooth with the exception of cuticle structures like setae, scales and combs. The cuticle differs only in thickness, which depends on the exposure of the different body regions. The cuticle of the penis and the cirrus must be thicker than the cuticle of the thorax, because these body parts are exposed to the environment and consequently they are an easy prey for predators.

In the depressions of the penis as well as between the cirri ridges are located. The ridges of the depressions are variable in their size, whereas the ridges of the cirri have all the same size. Nevertheless the ridges of both body regions may cause a certain rigidity and stiffness.

The cuticle of the degenerating penis with its crinkles is “mysterious”. An attempt of interpretation is made by the two hypotheses given explained above. Fig. 26 is also difficult to interpret. It has a cuticle in three zones: the inner is undulated, the middle is straight and the outer is crinkled. This is only detected on the basis of the penis of one individual. Maybe the inner part of the cuticle is not as elastic as the middle part and so it remains undulated after expansion during copulation. Another hypothesis indicates that a new and wider cuticle is accumulated, but the old cuticle is not shed. Therefore the new one forms undulations, because of the restriction of the old cuticle.

This study is the first, which investigates exactly the cuticle of the mature and degenerating penis as well as the cuticle of the cirri and the thorax of the cirripede *Semibalanus balanoides*. The thickness, the surface structures and the layering of the cuticle of the different body regions are compared and discussed. This information is the basis for further investigations.

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DANKE!

7. Curriculum vitae

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Studium

10/99 – 05/03	Biologie an der Universität Wien
05/03 – 10/08	Zoologie an der Universität Wien
03/07 – 10/08	Diplomarbeit zum Thema: Cuticle structures on different body regions of *Semibalanus balanoides* (L.) (Thoracica, Cirripedia) with special emphasis of mature and degenerating penis

Berufspraxis

01/04 – 05/04	Präparation und Bestimmung von Knochenfunden aus Vösendorf im Naturhistorischen Museum (Abt. Archäologisch-Zoologische Sammlung) im Auftrag des Bundesdenkmalamtes
10/04 – 12/06	Präparation und Bestimmung von Knochenfunden aus der Siedlung von Bruckneudorf im Naturhistorischen Museum (Abt. Archäologisch-Zoologische Sammlung) im Auftrag des Bundesdenkmalamtes
01/07 – 06/07	Präparation und Bestimmung von Knochenfunden aus Sarasdorf im Naturhistorischen Museum (Abt. Archäologisch-Zoologische Sammlung) im Auftrag des Bundesdenkmalamtes
10/07 – 02/08, 03/08 – 07/08, 10/08 – 02/09	Tutorin an der Einrichtung Cell Imaging und Ultrastrukturforschung der Fakultät für Lebenswissenschaften (Projektpraktikum: Submikroskopische Anatomie und Präparationstechnik)

Publikation

PUCHER, E., BRUCKNER, T., BAAR, A., DISTELBERGER, G., ÖHLINGER, B. ZHEDEN, V. (2007): Tierskelette und Tierknochen aus dem awarischen Gräberfeld von Vösendorf-Laxenburgerstraße. Fundberichte aus Österreich 45 (2006), 481 – 521. Wien.