

MASTERARBEIT

Titel der Masterarbeit

„Morphological characterisation of the cuticle of *Ibla cumingi*, Darwin, 1851 (Crustacea: Cirripedia) and differentiation of the cuticle and cement with histochemical methods“

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2015

Studienkennzahl lt.

A 066 831

Studienblatt:

Studienrichtung lt.

Masterstudium Zoologie UG2002

Studienblatt:

Betreut von:

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Summary

Ibla cumingi lives in the intertidal zone and therefore is exposed to strong currents. Like other Pedunculata this species use proteinaceous cement for attachment. The cement is used by the females to attach to the substratum and by the dwarf males to anchor inside the mantle cavity of the female.

Since the amount of produced cement is small, it is assumed that the cuticle supports attachment. To determine the involvement of the cuticle in adherence, the attachment regions of *Ibla cumingi* specimens were studied using light-and electron microscopy. On the peduncle base of female *Ibla cumingi* lobular cuticular structures were found which obviously grow into substrate crevices. Additionally, to the attachment via cement by the male on the female the tissue of the female also constricts around the antennules of the male. Both cases support the hypothesis that the attachment in male and female is chemical and physical.

Lobular structures were also found on the base of the peduncle in hermaphrodites of *Scalpellum scalpellum*. Also a species with scarce amounts of cement.

Immunogold labeling of the cuticle allowed differentiation between cuticle and cement. Closer inspection of the peduncle of the female *Ibla cumingi* revealed cuticular projections with a different morphology than bristles found on other Crustaceans. This morphology was observed closer in this work. They consist of cuticular substructures surrounded by a cuticular sheath. On the peduncle of *Scalpellum scalpellum* bristles as well as calcareous spines were present. Observing the peduncle of *Pollicipes pollicipes* no bristles only calcareous spines were observed.

Key words: cuticle, bristles, Cirripedia, *Ibla cumingi*, immunogold labeling, cement, attachment;

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1 Introduction

Over several years barnacles were in the focus of many investigations because of their role in biofouling (Callow and Callow, 2002). Their mode of attachment was of special interest (Kamino, 2013). Particularly possible application of the adhesive in medicine and technology found growing interest (Gohad et al., 2014). The amount of cement (adhesive) produced by the different species varies. *Dosmia fascicularis* (Ellis & Solander, 1786) a highly specialized barnacle produces the greatest amount of cement, which it uses as a float on the ocean surface (Zheden et al., 2012). Contrary to that only traces of cement, which are not visible macroscopically, are used by pedunculate species like *Ibla cumingi* Darwin, 1851, *Pollicipes pollicipes* (Gmelin, 1790) and *Scalpellum scalpellum* (Linnaeus, 1767). A comparative study of the fine structure of the adhesives of these three species was made by Rodharth (2013).

Ibla cumingi a gonochoristic species is considered basic within the Cirripedia (Burmeister, 1834) Thoracica (Darwin, 1861), (Klepal, 1984) and Anderson (1994) even created the superorder Prothoracica for the Ibloidea. *Ibla cumingi* is an intertidal marine animal, living on rocks and shell plates of the acorn barnacle *Tetraclita squamosa* (Bruguière, 1789).

Pollicipes pollicipes is also an intertidal barnacle. It often occurs together with *Mytilus californianus* covering rocks and artificial structures (Barnes and Reese, 1960). Depending on wave exposure, *Pollicipes* sp. attaches either with a short peduncle and a broad base or in less exposed areas with an elongate peduncle and a small base (Barnes and Reese, 1960). Due to its special location in the surf zone *Pollicipes pollicipes* developed new ways of attachment. The cuticle near the base of the peduncle may form lobes which additionally increase the area of attachment. Apart from that these

structures may contribute to movement away from the initial place of their attachment (Kugele & Yule, 1993).

Because of the attachment of *Ibla cumingi* within crevices and by the enlargement of the peduncular base of *Pollicipes pollicipes* it can be speculated that the mode of adherence in these two species may be both chemical and mechanical. The dwarf males of *Ibla cumingi* attach inside the mantle cavity of the female. The question arises whether the males also show indications of a combined chemical and mechanical attachment to the female tissue. In *Ibla cumingi* and *Pollicipes pollicipes* it is sometimes difficult to distinguish between cuticle and cement both macroscopically and by light microscopy. In this context it seemed important to find a reliable method for the distinction of these matrices. For that purpose light microscopy, electron microscopy and immunotechniques were applied.

A comparative morphological study of the structure and dimensions of the cuticle and its projections in females and males of *Ibla cumingi* was also in the focus of interest.

For a comparison of the peduncular projections *Scalpellum scalpellum* is also considered in this study. It occurs from shallow to deeper waters (20 m - 200 m). The individuals attach to rock surfaces, hydroids like *Tubularia indivisa* (Linnaeus, 1758) or *Nemertesia antennina* (Linnaeus, 1758), (Buhl-Mortensen and Hoeg, 2006) and to spines of sea urchins *Cidaris cidaris* (Buhl-Mortensen, Hoeg and Yusa, 2012).

2 Materials and Methods

Specimens

6 individuals of *Ibla cumingi* (Darwin, 1851) were collected 2012 from the shells of *Tetraclita* sp. (Schumacher, 1817) in the Gulf of Eilat. They were

kept in a seawater tank for 4 months and fed with nauplii of *Artemia* sp. and algae (Hobby Mikrozell 15ml Dohse Aquaristik, Otto-Hahn Straße9, D-53501 Grafschaft Gelsdorf). The salinity of the water was controlled with a hydrometer. For comparison specimens of *Pollicipes pollicipes* (Gmelin, 1790) and of *Scalpellum scalpellum* (Linnaeus, 1767) were used. 2 specimens of *Pollicipes pollicipes* (Gmelin, 1790) were collected in 2010 from Cabo de Sines, Portugal. These animals were already embedded in Low viscosity resin blocks. 2 specimens of *Scalpellum scalpellum* (Linnaeus, 1767) were collected in 2012 from *Cidaris cidaris* (Linnaeus, 1758) spines in Norway during the MAREANO-program by Dr. Lene Buhl-Mortensen. They were first preserved in formaldehyde and later kept in ethanol. Sample R1044 comes from position: 62°28.14'N 4°28.05'E in a depth of 214 m and sample R1046 was found at the position: 62°22.56'N 4°27.03'E in a depth of 194 m.

For SEM investigations

One gold-coated female of *Ibla cumingi* was used. 3 randomly selected cuticular projections on the peduncle were measured.

Sample preparation for TEM investigations

The bodies of 4 *Ibla cumingi* females cut into pieces and 4 males separated from the females and cut in half were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide at a pH of 7.4 in 0.1 mol cacodylate buffer with sucrose (10%) and dehydrated in ethanol. Acetonitrile was used as an intermedium. For structure analysis the samples were embedded in low viscosity resin. For lectin staining osmium tetroxide was omitted in the fixation protocol and the samples were embedded in LR-white. Parts of the peduncle of *Scalpellum scalpellum* were fixed in 2.5% glutaraldehyde (5h),

rinsed 3 times(10min each) and then put into EDTA 0.1 M (5h) to dissolve the calcium of the plates. After washing in 0.1mol cacodylate buffer with sucrose (10%), the samples were dehydrated in ethanol and embedded in LR-white for lectin staining. Semithin sections of 500nm were cut with a Leica Ultracut-S microtome and a Leica EM UC7, stained with toluidine blue and examined with a light microscope (Olympus BX41). For electron microscopy sections of 60 to 70nm thickness were cut. They were stained with 0.5% uranyl acetate and 3% lead citrate. The ultrathin sections were viewed with transmission electron microscopes (Zeiss EM 902 (80kV) and Zeiss Libra 120 (120kV)). The gold coated (by an Agar B7340 sputter coater) *Ibla cumingi* sample was examined with scanning electron microscopes Philips XL 20 (15 kV) and JEOL JSM-IT300 (JEOL (Germany) GmbH, München, Germany). With the program iTEM Imaging Platform (Olympus Soft Imaging Solutions GmbH, Münster, Germany) the TEM images were viewed. To work on the images Photoshop CS5 (Adobe Systems Inc., USA) was used. For statistical analysis to acquire the thickness of the cuticle in different planes of the section Excel 2010 was used.

Measurement of the cuticle of the body, peduncle and cirri

The cuticle of the cirri, body of male and female and the peduncle of *Ibla cumingi* was measured and the thickness of epi- and procuticle was obtained individually as well as together in all cases. Whereas epi-, exo- and endocuticle can be distinguished clearly in the cirri these cuticle layers melt into each other on the rest of the body cuticle as well as on the cuticle of the peduncle. On the peduncle three regions were investigated more closely. (For orientation the peduncle was divided into three regions. The regions are as follows: the basal part, where the animal attaches to the substratum, the middle and the upper part just below the scuta and terga. The body

cuticle of the female was measured in the region near where the male attaches to the female inside the mantle cavity. The body cuticle of the male was measured distal of the antennules after these were removed in the region where sperm can already be seen inside the male.

Measurement of the cuticular projections on the peduncle of the female of *Ibla cumingi*

For TEM measurements 60nm thick cross-sections of the cuticle of the peduncle were used to randomly select 9 cuticular projections. These projections are ovoid in cross section and one side is more sinuate than the other (Fig.3, 10). The diameters of 3 randomly selected samples of each region were measured (Fig.3). Each projection contains a number of substructures which were counted on the sections in the TEM. Each substructure consists of concentric cuticle layers which were counted. The thickness of the female cement was also measured (n=20). Also the fiber thickness in the matrix of the cement was measured (n=30).

Immuno gold staining

The following reagents were used

As primary antibody biotinylated *Maackia amurensis* Lectin II (MAL II) (Vector B-1265) 1mg/ml in a dilution of 1:50 (2 μ l) and also biotinylated *Wheat germ* Agglutinin (Vector B-1025) 5mg/ml in 1ml H₂O in a dilution of 1:500 (2 μ l) was used. MAL II binds to carbohydrate structures and contains sialic acid (Knibbs, Goldstein et al., 1991) and WGA binds for N-acetylglucosamine and N-acetylneuraminic acid (Park, C., et al, 2014). As secondary antibody Rabbit IgG α Biotin (Abcam53494) LotGR31094-2, 1mg/ml in glycerol/PBS 1:1 in a dilution of 1:200 (5 μ l) was used. For gold

labeling either Protein A- 10nm gold conjugate or Goat α Rabbit IgG-12nm Gold (Jackson Bioxxx 111-205-144) in a dilution of 1:20 (5 μ l) was used. As blocking solution 0,5% gelatin from cold water fish skin (FSG) (G7765 SIGMA) in PBS was used.

Indirect method

60nm thick sections of the cuticle and cement at the base of the peduncle of *Ibla cumingi* and of *Scalpellum scalpellum* were used for immunogold staining. The cuticle of the cirrus of *Ibla cumingi* served as negative control. Formvar coated nickel grids were transferred from drop to drop containing either antibody or rinsing solution. The series was started with FSG (fish skin gelatin) as blocking agent to inactivate residual aldehyde groups present after aldehyde fixation, followed by PBS (phosphate-buffered saline). As primary antibody 6 grids were labelled with MAL II (*Maackia amurensis* lectin) (20 min.) 6 grids were labelled with WGA (wheat germ agglutinin) (20 min.) followed by rinsing with PBS and 1% FSG. The secondary antibody rabbit anti biotin was applied and rinsed with PBS. This was then followed by the tertiary antibody goat α rabbit IgG-12nm gold (20min). All grids were rinsed with PBS and water. All rinsing steps took about 3 min. each. The sections were stained with 0.5% uranyl acetate (5 min.). After air-drying the grids were examined with a Zeiss Libra 120 transmission electron microscope (120 kV). To determine non-specific binding of lectins to the tissue negative controls of identical sections were processed according to the same methods with the primary antibody replayed with PBS.

Direct method

2 Grids with sections of 60nm on nickel formvar grids containing female *Ibla cumingi* cement and cuticle were put directly on a WGA+10nm gold mix (WGA colloidal gold 10 nm, Colloidal gold conjugated Triticum vulgare lectin, LOT 310516, EY labor inc. USA). No secondary antibody was used.

3 Results

Measurements

Measurements of the cuticle of *Ibla cumingi*:

The cuticle of *Ibla cumingi* females varies in thickness on different body regions of the animal. Also endo-, exo- and epicuticle can clearly be distinguished only on the cirri (Fig.1), which was not possible on the body cuticle of the female, the cuticle of the peduncle of the female and not on the body cuticle of the male. On these body parts only epicuticle and procuticle are discernable (Fig.2). The contact zone of the cuticle with the substratum is deeply lobed (Fig.3). At the base of the peduncle the procuticle is about 299 μm and the epicuticle is about 46 nm thick. (Fig.2) In the middle part of the peduncle (cuticle thickness 227,41 μm) the procuticle measured 227.37 μm and the epicuticle 38,25 nm. Directly below the opercular plates the cuticle of the peduncle measured 354,20 μm consisting of the procuticle with a thickness of 354,18 μm and an 38,25 nm thick epicuticle (Tab. 2). Based on cross sections of the rami of a cirrus we can differentiate between two short and two long sides (Fig.3). On the short sides the cuticle measured 6,58 μm and on the long sides it measured about 7,53 μm . The epicuticle measured 0,12 μm on the shorter side and 0,11 μm on the longer side. The procuticle on the short side was about 6,45 μm while the

procuticle on the long side was about 7,41 μm thick. The exocuticle on the short side of the cirrus measured 1,55 μm and on the long side 1,58 μm . The endocuticle had a thickness of 4,90 μm on the short side and 5,83 μm on the long side.

On the body of the *Ibla cumingi* male only epi- and procuticle can be clearly distinguished. In comparison with the body cuticle of the female (2.7 μm thick) the cuticle of the *Ibla cumingi* male is considerably thicker (4.0 μm) (Tab. 3).

The cement of the female used for attachment on the substratum is about 1,25 μm thick (Tab. 4).

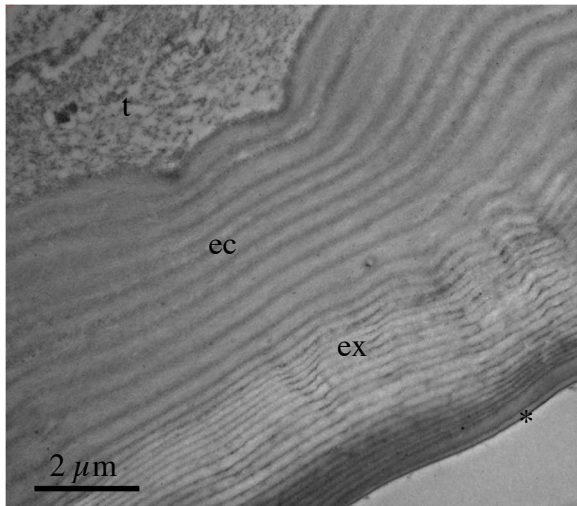


Fig.1: TEM of the cirrus of the female of *Ibla cumingi*. The layers of the cuticle, endo- (ec), exo- (ex) and epicuticle (*) are visible. On the inside we see tissue (t).

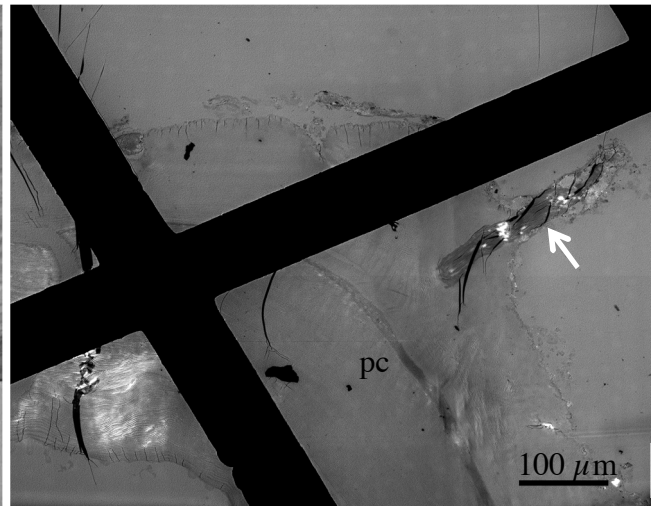


Fig.2: TEM *Ibla cumingi*: peduncle of the female. The cuticle of the peduncle (pc) is not differentiable into endo and exocuticle. (arrow) longitudinal section of a cuticular projection.

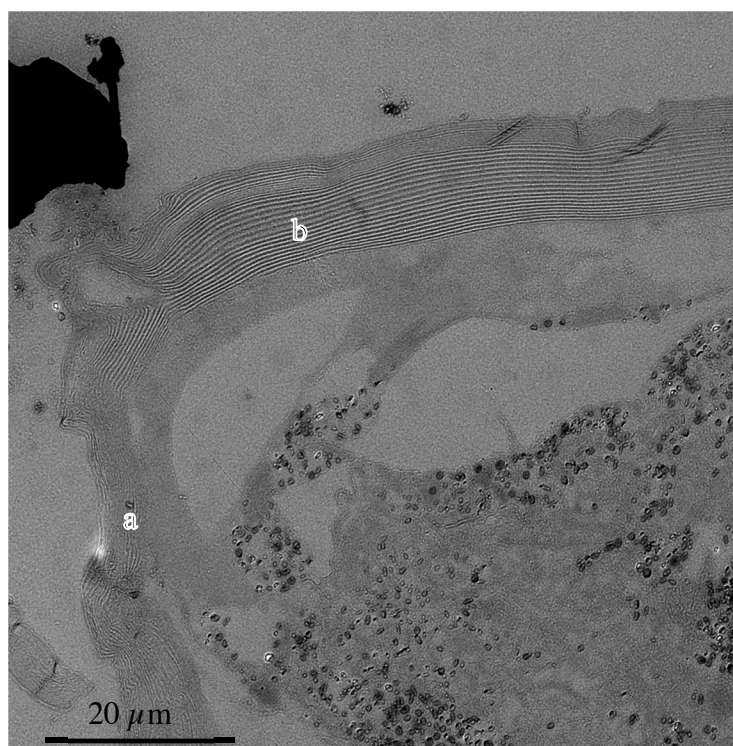


Fig.3: TEM *Ibla cumingi*: Cirrus of the female, (a) short side and (b) long side.

Tab. 1: TEM measurements of *Ibla cumingi*: thickness of the cuticle on the base, middle and upper part of the peduncle of the female (standard deviation values in brackets):

		Peduncle		
		basal part	middle part	upper part
	Number of Measurements	20	20	20
Mean	Cuticle thickness	299,55 μm (107,33)	227,41 μm (21,81)	354,20 μm (29,69)
	Epicuticle	46,00 nm (0,07)	33,91 nm (0,003)	38,25 nm (0,003)
	Procuticle	299,50 μm (107,36)	227,37 μm (21,81)	354,16 μm (29,71)
Min	Cuticle thickness	128,74 μm	187,33 μm	296,94 μm
	Epicuticle	21,43 nm	12,93 nm	16,03 nm
	Procuticle	128,70 μm	187,29 μm	296,90 μm
Max	Cuticle thickness	485,20 μm	294,77 μm	415,62 μm
	Epicuticle	83,66 nm	63,58 nm	63,58 nm
	Procuticle	485,16 μm	294,74 μm	415,58 μm

Tab. 2: TEM measurements of the thickness of cirrus cuticle of the *Ibla cumingi* female (standard deviation values in brackets):

	Number of Measurements	Cirri [μm]	
		short side	long side
		20	20
Mean	Cuticle thickness	6,58 (2,34)	7,53 (1,49)
	Epicuticle	0,12 (0,10)	0,11 (0,01)
	Exocuticle	1,55 (0,14)	1,58 (0,57)
	Endocuticle	4,90 (2,43)	5,83 (1,35)
Min	Cuticle thickness	3,64	3,01
	Epicuticle	0,02	0,03
	Exocuticle	1,28	0,91
	Endocuticle	1,86	1,99
Max	Cuticle thickness	11,22	9,21
	Epicuticle	0,49	0,18
	Exocuticle	1,86	2,30
	Endocuticle	9,88	7,86

Tab. 3: TEM measurements of the body cuticle of *Ibla cumingi* male and female (standard deviation values in brackets):

	Number of Measurements	male [μm]	female [μm]
		body cuticle	body cuticle
		20	20
Mean	Cuticle thickness	4,00 (1,09)	2,69 (0,42)
	Epicuticle	0,04 (0,01)	0,31 (0,56)
	Procuticle	3,96 (1,09)	2,37 (0,69)
Min	Cuticle thickness	2,63	1,89
	Epicuticle	0,14	0,04
	Procuticle	2,6	0,1
Max	Cuticle thickness	8,21	4,54
	Epicuticle	5,67	3,25
	Procuticle	8,15	4,36

Tab. 4: TEM measurements of the thickness of the cement of *Ibla cumingi* female which is used for attachment to the substratum (standard deviation values in brackets):

	female cement thickness [μm]
Number of Measurements	20
Mean	1,25 (0,56)
Min	0,08
Max	2,36

Measurements of the cuticular projections found on the peduncle of the female of *Ibla cumingi*:

Cuticular projections are scanty scattered on the base of the peduncle. Where the peduncle attaches to the substratum only one projection may be seen (Fig.2). Towards the opercular plates the distribution of cuticular projections gets denser (Fig.4, 5).

The cuticular projections on the peduncle of the female have inner substructures (Fig. 4, 8). The diameter of each subunit is around 5 μm (Tab.5). The number of the subunits in several cuticular projections was counted (Tab.5, 6, 7). On the outside of the cuticular projection is a sheath which surrounds the subunits (Fig.7, 9). Inside there are cuticular rings which make up the cuticular subunits (Fig.7, 10, 11). On the cross sections of the subunits, open and closed rings of the three cuticular layers were counted on the basal part (Tab. 5), on the middle part (Tab. 6) and on the upper part of the peduncle (Tab. 7). Some of the rings forming the subunits in the cross sections are completely closed others are open where they touch a neighboring subunit. The subunits of the cuticular projections have a diameter around 5 μm , (measured on SEM and TEM). The cuticular projections were divided into three regions (A, B, C) (Fig.4) to investigate the form of the different subunits as they are not identical (Tab.5, 6, 7). The subunits in the middle of the cuticular projections show less cuticular rings than those closer to the cuticular sheath. Subunits closer to that sheath

show most closed rings. The open rings seem to stop when coming in contact with another subunit. The cuticle forming those ring is about 100 nm thick no matter where the thickness of cuticular rings forming the subunits was measured.

The epithelial cells produce the cuticle and its cuticular structures (Fig. 12, 13, 14, 15, 16, 17). The subunits are produced by cells (Fig.12, 15) forming cone like tips which extend (Fig.14). The cuticular projections taper from the base to the distal end and show regular constrictions every 300 μm . In *Scalpellum scalpellum* only bristles were present on the peduncle (Fig.18, 19). On the peduncle of *Pollicipes pollicipes* no structures like the projections of *Ibla cumingi* could be discerned.

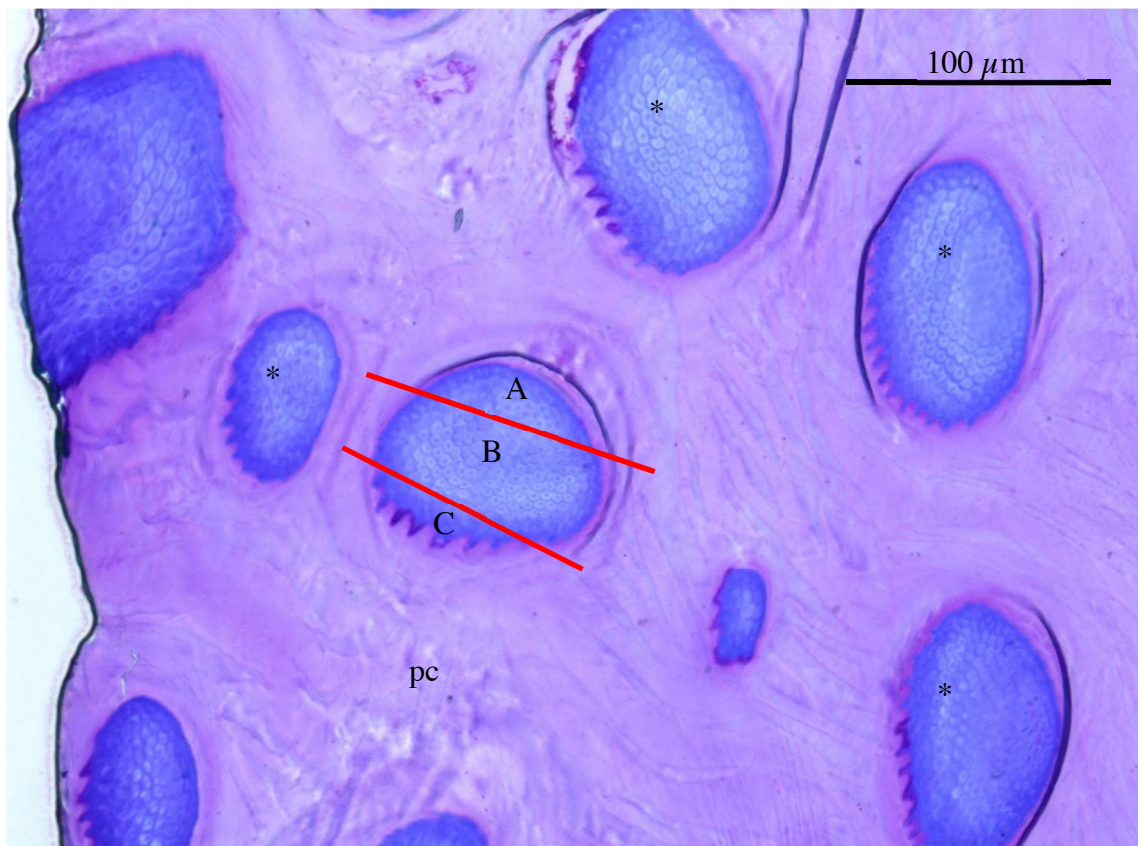


Fig.4: *Ibla cumingi*; female. Peduncle (pc) near the opercular plates, intersected with cuticular projections (*). The red lines show the subdivisions that were used for the measurements. (A) upper end, (B) middle part, (C) lower part of the cuticular projection.

Tab.5: TEM measurements of the projections basal where the female *Ibla cumingi* attaches to the substratum. Size of the projection, number of subunits like, number of open and closed rings inside each substructure, diameter of three (A, B, C) subunits (standard deviation values in brackets):

Projection I											
size [μm]		upper part			middle part			lower part			
a	27,3	A	B	C	A	B	C	A	B	C	
b	32,95										
Substructures [n]	25										
Rings [n]	open	11	7	5	1	3	3	9	7	15	
	closed	0	0	0	1	1	0	2	2	0	
Ringsize [μm]	a	5,95	4,21	6,54	8,21	7,5	6,72	6,73	7,19	7,22	
	b	5,41	5,27	4,23	4,44	4,46	4,81	6,04	5,28	4,79	
Ringsize Mean											5,83 (1,22)
homogen		4,77	4,08	5,47	5,37	4,76	5,52	0,86	0,62	3,39	
Projection II											
size [μm]		upper part			middle part			lower part			
a	49,1	A	B	C	A	B	C	A	B	C	
b	52,25										
Substructure [n]	70										
Rings [n]	open	14	7	16	6	8	21	4	9	9	
	closed	0	3	1	0	0	0	1	0	0	

Ringsize[μm]	a	6,4	4	4,89	6,75	6,16	7,67	2,73	4,99	3,59	
	b	3,51	2,74	4,1	6,02	6,43	5,3	4,93	5,07	4,09	
Ringsize Mean											4,97 (0,39)
homogen		1,7	1,25	1,68	4,83	4,37	3,72	2,53	3,07	1,76	
Projection III											
size [μm]		upper part			middle part			lower part			
a	57,01	A	B	C	A	B	C	A	B	C	
b	66,64										
Substructures [n]	57										
Rings [n]	open	17	21	28	28	16	17	17	11	20	
	closed	2	3	4	0	8	5	9	0	6	
Ringsize [μm]	a	5	7,04	4,87	5,77	5,71	6,44	4,94	5,69	6,82	
	b	5,7	5,16	3,64	4,79	4,08	4,64	4,51	5,07	4,63	
Ringsize Mean											5,25 (0,79)
homogen		1,78	0,53	0,7	-	-	0,47	-	1,97	0,76	

Tab.6: TEM measurements of the projections in the middle of the peduncle of the female *Ibla cumingi*. Size of the projection, number of subunits, number of open and closed rings inside each subunit, diameter of three (A, B, C) subunits (standard deviation values in brackets):

Projection I											
size [μm]		upper part			middle part			lower part			
a	74,45	A	B	C	A	B	C	A	B	C	
b	97,21										
Substructures [n]	168										
Rings [n]	open	4	2	2	4	5	6	7	14	9	
	closed	14	8	17	1	0	0	6	6	3	
Ringsize [μm]	a	6,2	5,83	5,63	5,48	5,31	5,5	4,61	4,43	5,31	
	b	5,47	5,58	6,56	5,1	5,86	5,63	5,71	5,86	5,16	
Ringsize Mean											5,51 (0,2)
homogen		0,7	3,69	1,68	3,84	4,36	4,34	1,14	1,08	2,52	

Projection II											
size [μm]		upper part			middle part			lower part			
a	51,73	A	B	C	A	B	C	A	B	C	
b	85,59										

Substructures [n]	126										
Rings [n]	open	13	12	13	9	11	5	10	10	15	
	closed	1	7	4	0	0	1	9	8	0	
Ringsize [μm]	a	5,77	6,57	6,82	4,33	6,52	4,72	4,15	5,47	4,19	
	b	5,38	4,48	4,1	6,73	4,7	6,18	6,86	4,65	5,83	
Ringsize Mean											5,41 (0,02)
homogen		3,39	0,63	2,05	3,92	4,002,45	3,97	0,86	0,27	3,06	
Projection III											
size [μm]		upper part			middle part			lower part			
a	55,59	A	B	C	A	B	C	A	B	C	
b	89,8										
Substructures [n]	141										
Rings [n]	open	13	11	9	6	12	11	8	11	14	
	closed	9	7	5	10	0	0	6	8	7	
Ringsize [μm]	a	6,2	5,14	4,64	4,87	5,36	5,2	3,61	5,32	6,02	
	b	4,32	5,03	4,32	5,82	5,27	5,37	2,71	2,71	4,48	
Ringsize Mean											4,8 (0,49)
homogen		-	-	-	4,6	4,15	4,71	-	-	-	

Tab. 7: TEM measurements of the cuticular projections in the upper part of the peduncle of the female *Ibla cumingi* where the peduncle reaches the opercular plates. Size of the projection, number of subunits, number of open and closed rings inside each subunit, diameter of three (A, B, C) subunits (standard deviation values in brackets):

Projection I											
size [μm]		upper part			middle part			lower part			
a	109,96	A	B	C	A	B	C	A	B	C	
b	79,45										
Substructures [n]	245										
Rings [n]	open	11	6	11	5	4	6	6	7	9	
	closed	0	0	0	0	0	0	10	7	4	
Ringsize [μm]	a	5,74	4,42	4,73	5,66	5,33	6,18	4,63	6,02	5,52	
	b	2,94	3,28	5,32	5,81	5,21	6,17	2,16	3,05	3,6	
Ringsize Mean											4,76 (0,83)
homogen		2,37	3,04	3,23	4,72	4,7	5,89	-	-	-	
Projection II											
size [μm]											
a	109,7	upper part			middle part			lower part			
b	70,99										
Substructures	181										

[n]											
Rings [n]	open	17	13	9	7	8	7	10	7	7	
	closed	9	4	2	0	0	0	7	9	7	
Ringsize [μm]	a	4,27	4,81	5,72	6,12	6,66	5,3	5,16	5,81	6,37	
	b	3,38	4,28	4,52	6,1	5,37	5,42	4,78	5,47	3,56	
Ringsize Mean											5,17 (0,57)
homogen		-	1,68	3,04	4,78	5,1	4,28	-	-	-	
Projection III											
size [μm]		upper part			middle part			lower part			
a	107,08	A	B	C	A	B	C	A	B	C	
b	76,12										
Substructures [n]	213										
Rings [n]	open	10	12	9	3	3	5	10	6	8	
	closed	0	0	4	0	0	0	0	7	8	
Ringsize [μm]	a	4,76	4,59	4,26	5,39	4,42	5,58	5,39	5,45	6,23	
	b	6,39	5,48	6,35	5,93	6,03	5,51	4,28	4,89	6,04	
Ringsize Mean											5,39 (0,37)
homogen		2,23	3,06	0,84	5,11	4,61	3,66	3,45	-	-	

Tab.8: SEM measurements of *Ibla cumingi* peduncle of the female, diameter (μm) of the subunits of the projections.

Projections	diameter (μm)
mean	5,3
standard deviation	0,39

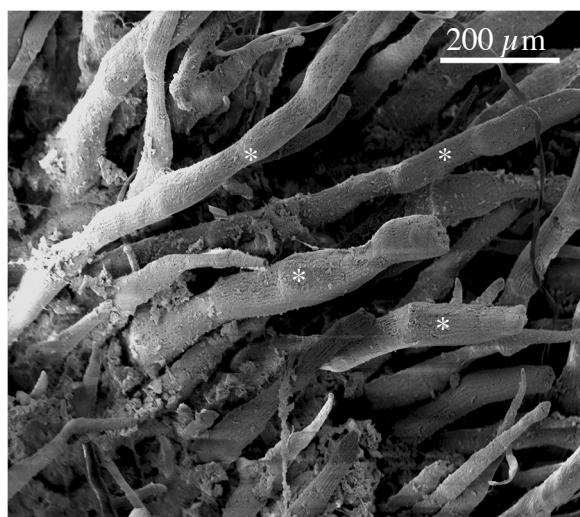


Fig.5: SEM: Cuticular projections (*) on the peduncle of the female of *Ibla cumingi*.



Fig.6: SEM: Detail of one cuticular projection (*) with its rough surface.

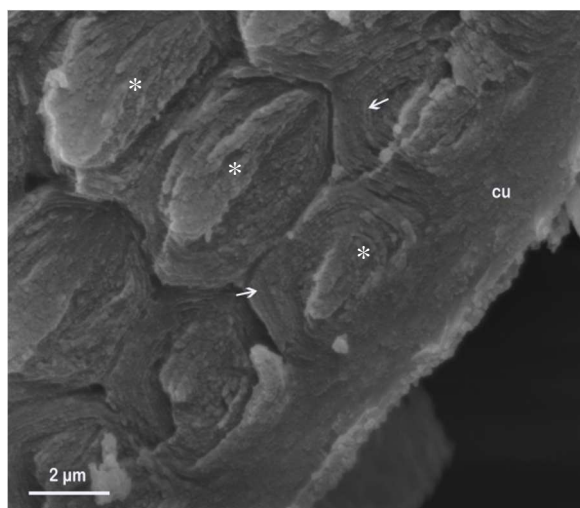


Fig.7: REM: detail of a cross section of one cuticular projection with subunits (*). Every subunit is made up by circular cuticle ring (arrow). These subunits are held together by a cuticular sheath (cu).

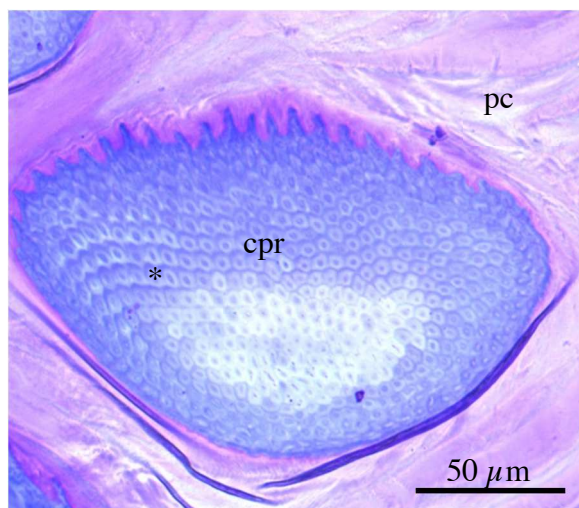


Fig.8: Cuticular projection (cpr) with subunits (*) surrounded by the cuticle of the Peduncle (pc).

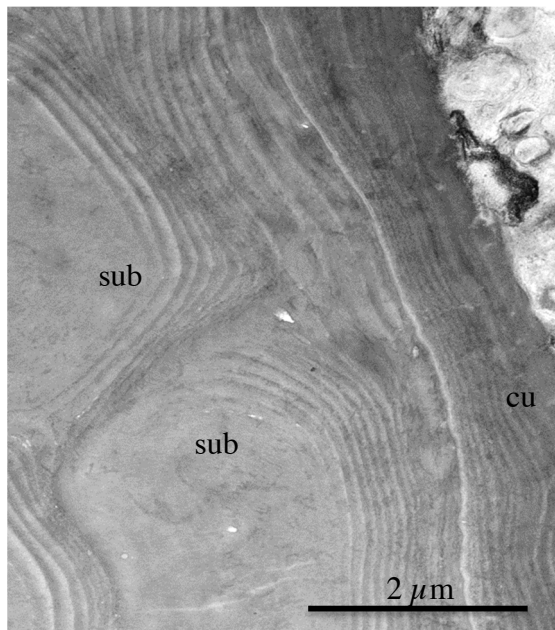


Fig.9: Detail of one cuticular projection. Margin with substructures (sub) and the cuticle sheath (cu) which surrounds the projection. Several cuticle layers that look homogenous, meaning individual ring-like cuticle layers can no longer be recognized as such, but as one area with feathery inclusions consisting of single cuticle strands, make up the inner part of each subunit.

Fig.10: Subunits with a circular form in the middle of the cuticular projection.

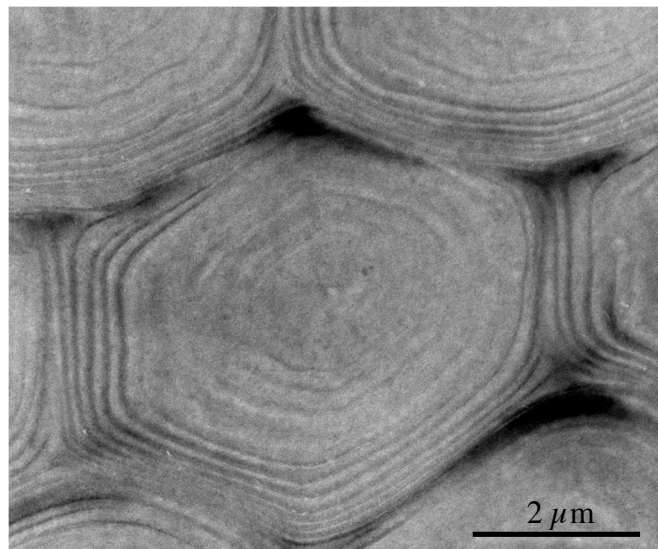
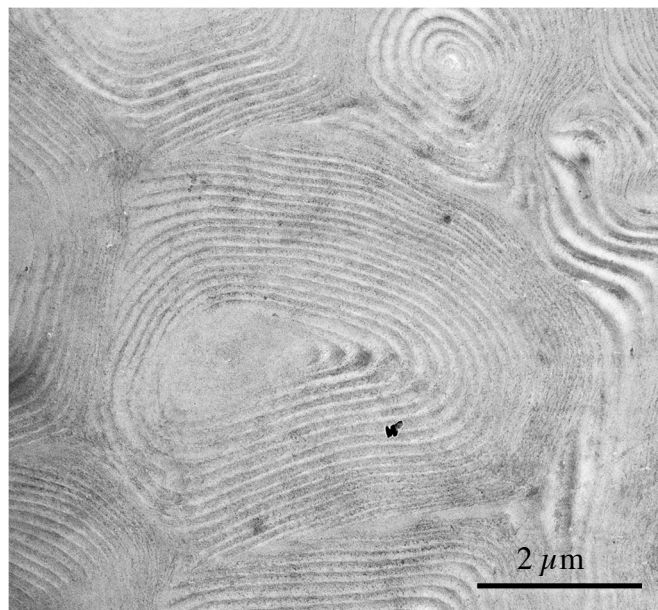


Fig.11: Subunits with an oval form near the margin of the cuticular projection.



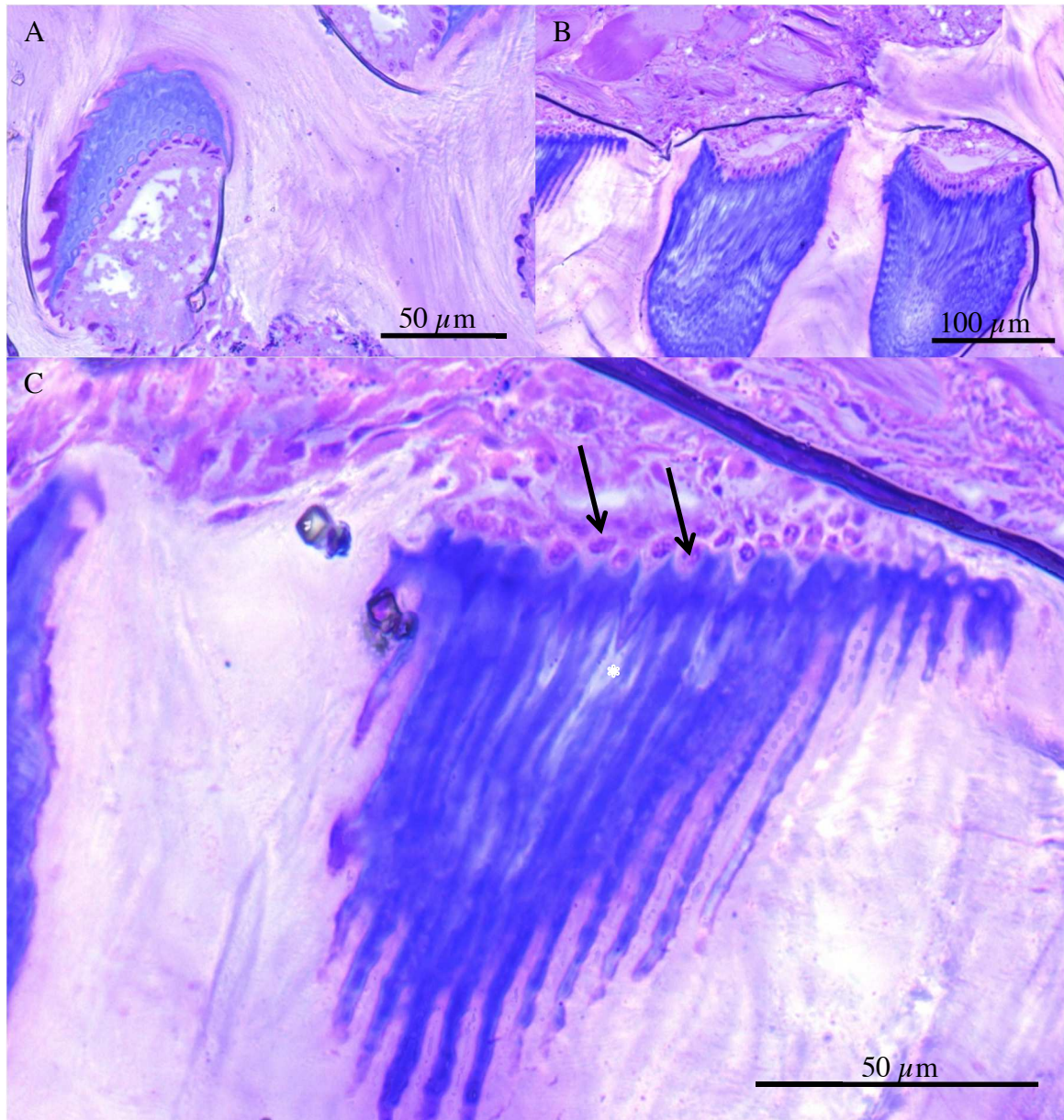


Fig.12: Cuticular projections: (A) longitudinal section with cell nuclei at the base where the subunits are being generated by the epithelial layer inside the cuticular projections (B),(C) longitudinal section of the subunits (*) with the nuclei at the base (arrows)

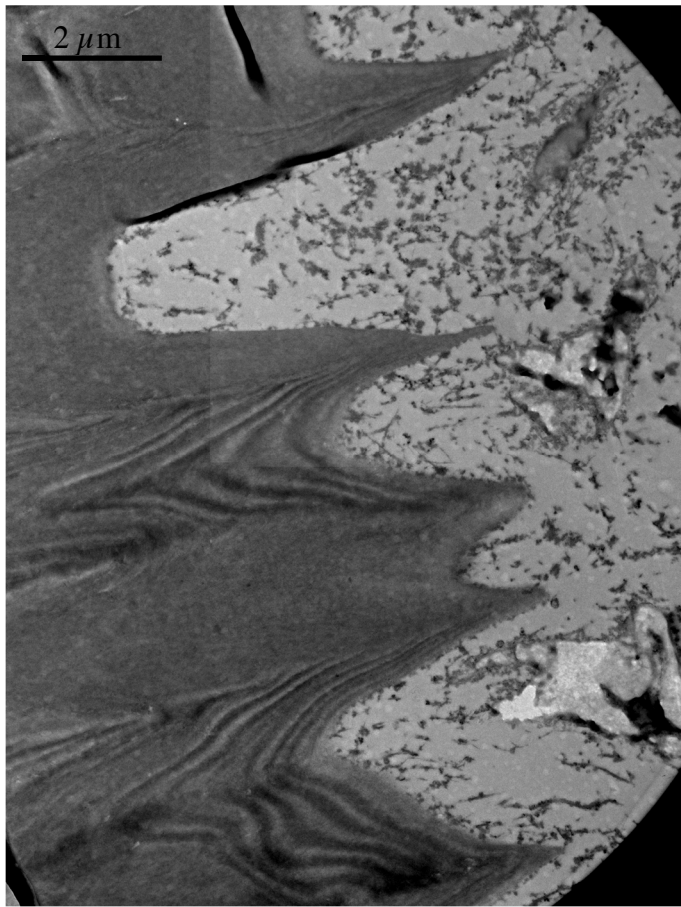


Fig.13: Cuticular projection basal where the subunits are formed. Tissue and cells are destroyed.

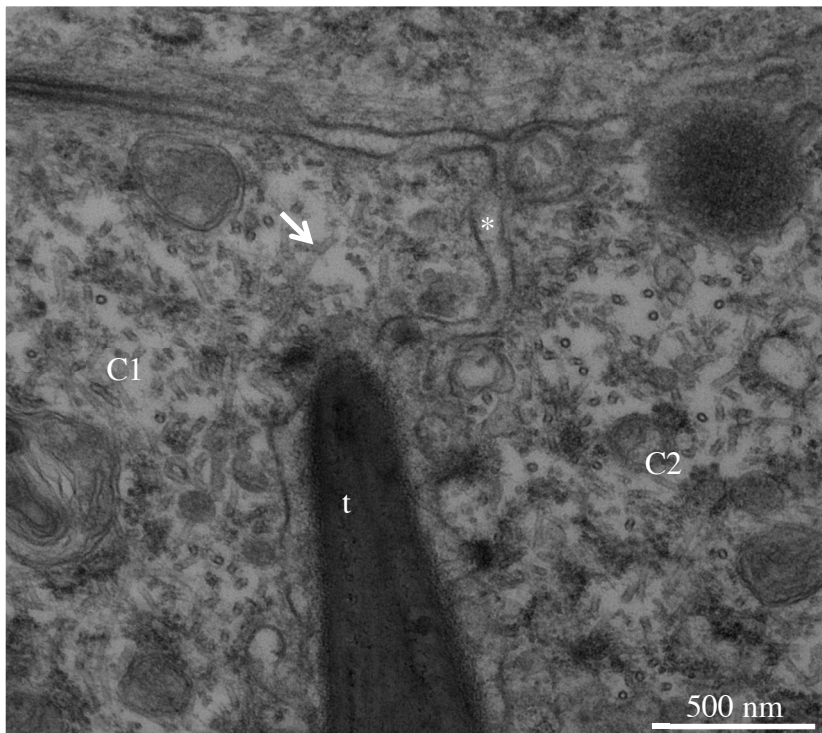


Fig.14: Tip (t) of the basal end of a subunit surrounded by lots of microtubules (arrow) seemingly growing within the intercellular space between (*) cell 1 (C1) and cell 2 (C2)

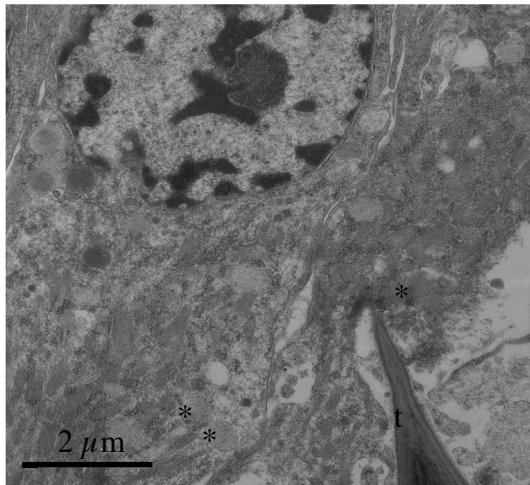


Fig.15: Nucleus of the cell forming the cuticular projections. Lots of mitochondria (*) surround the tip of the subunit (t).

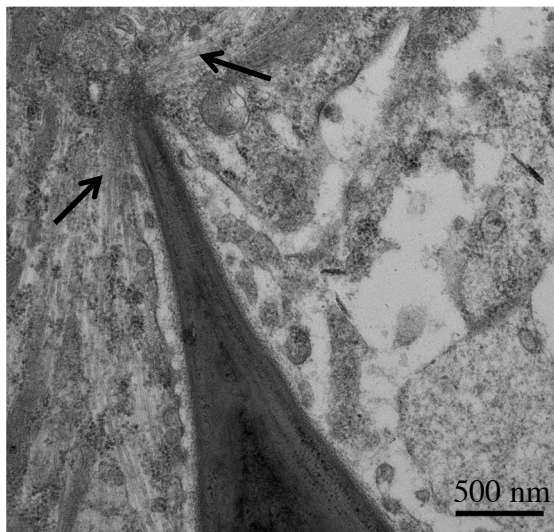


Fig.16: detail of microtubules that run to the tip of the subunit from 2 sides (arrows)

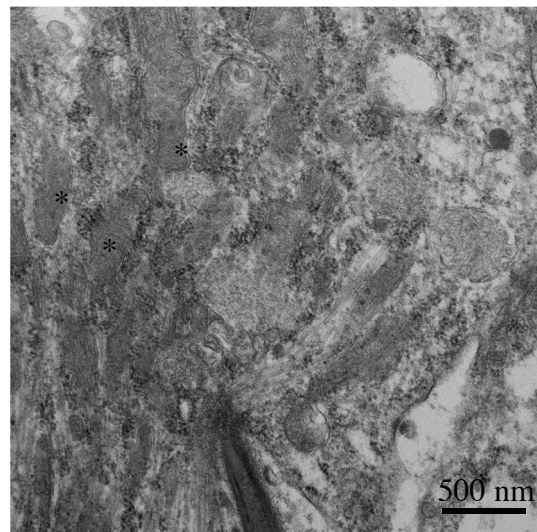


Fig.17: Beside lots of microtubules that run around the tip there are also lots of mitochondria (*).

Bristles of *Scalpellum scalpellum*

Bristles on the basal part of the peduncle of *Scalpellum scalpellum* where it attaches to the substratum (Fig.18, 19). These bristles are hollow inside with a broad base and ending in a tip.

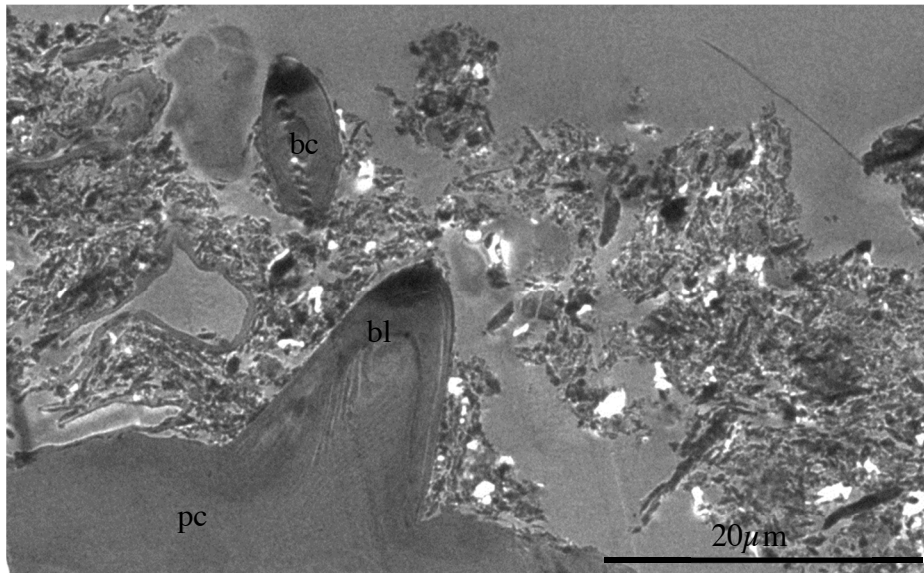


Fig.18:
Scalpellum scalpellum:
cuticle of the
peduncle (pc)
with bristles in
longitudinal
section (bl) and
bristles in
cross-section
(bc).

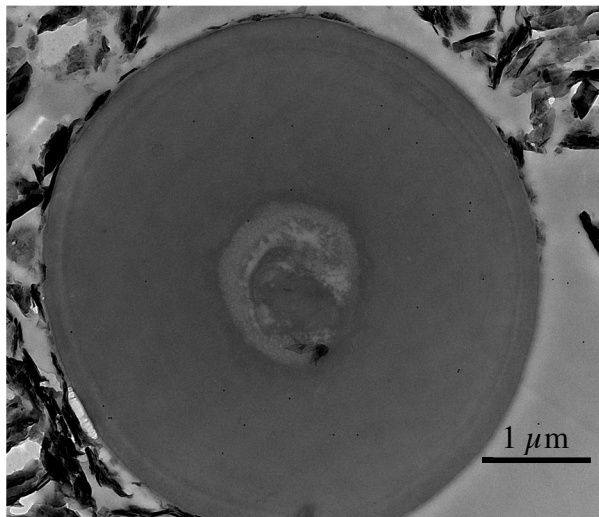


Fig.19: cross section
of a bristle on the
peduncle of
Scalpellum scalpellum.

Cement

Light microscopy

Cement can be seen by light microscope as a homogenous mass on the base of the female or between the antennules of the male. (Fig.20). The male is stuck to the female with the antennules and the female tissue surrounds the basal part of the peduncle. In the male the antennules are embedded in cement, which is surrounded by cuticle of the female in several layers.

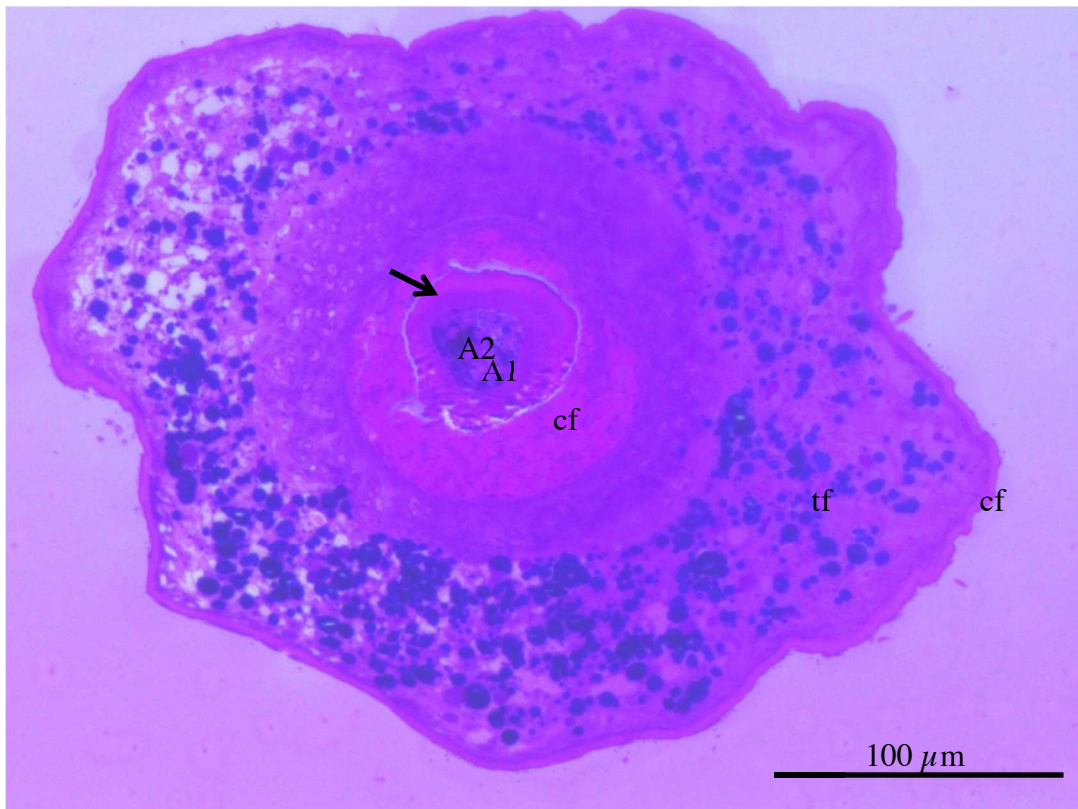


Fig.20: *Ibla cumingi*. male embedded inside the female. Male cuticle (arrow). Antennules one and two (A1 and A2), female cuticle (cf) and female tissue (tf).

Electron microscopy

Cement previously seen as a homogenous mass on light microscopic level shows a matrix with bundles of embedded fibers on electron microscopic level (Fig 26). Often the distinction or border between cement and cuticle is better seen on electron microscopic level (Fig.25) but there are cases where the distinction is nonetheless difficult. In these cases histochemical methods can be applied (Fig.23).

Indirect method

Ibla cumingi cirrus (Fig.21) and cuticle (Fig.23, 25) of the female and also the cuticle (Fig.26, 27) of the male were labeled with the primary AB, WGA followed by the secondary AB, Rabbit anti Biotin and 12nm gold. The lectin stains the procuticle of the animals. No difference in intensity of the stain was observed. WGA was also able to stain the procuticle of *Scalpellum scalpellum* (Fig.28). Whereas the primary AB WGA was exchanged with MAL II the results showed no stain on the procuticle, neither on the cirrus (Fig.22) nor on the cuticle of the peduncle (Fig.24). Cement is recognized by bundles where the matrix in which these are embedded is washed away (Fig.23, 24, 25, 26, 27, 28). The composition of the bundles is seen in female cement (Fig.23, 24, 25), male cement (Fig.26, 27) of *Ibla cumingi* as well as in the cement of *Scalpellum scalpellum* (Fig.28).

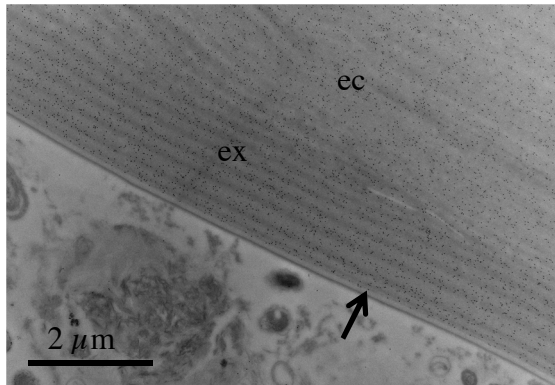


Fig.21: *Ibla cumingi* female: Cirrus labeled with WGA, Rabbit anti Biotin, 12nm gold. Epicuticle (arrow), exocuticle (ex), endocuticle (ec).

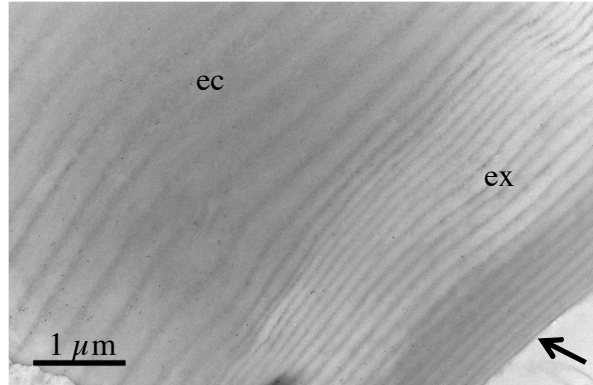


Fig.22: *Ibla cumingi* female: Cirrus labeled with MAL II, Rabbit anti Biotin, 12nm gold. Epicuticle (arrow), exocuticle (ex), endocuticle (ec).

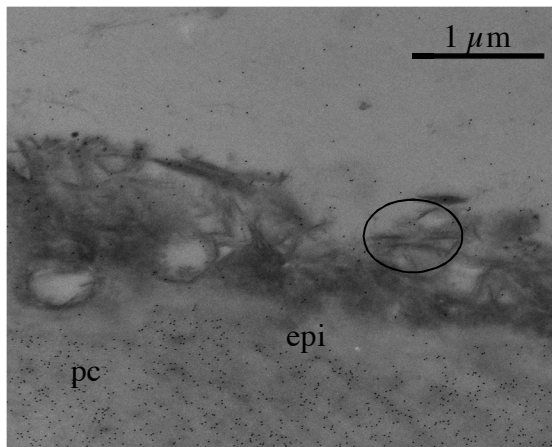


Fig.23: *Ibla cumingi* female: Attachment area of the peduncle to the substratum. Procuticle (pc) labeled with WGA, Rabbit anti Biotin, 12nm gold. Epicuticle (epi), no label. Cement bundles (circle) on the outside.

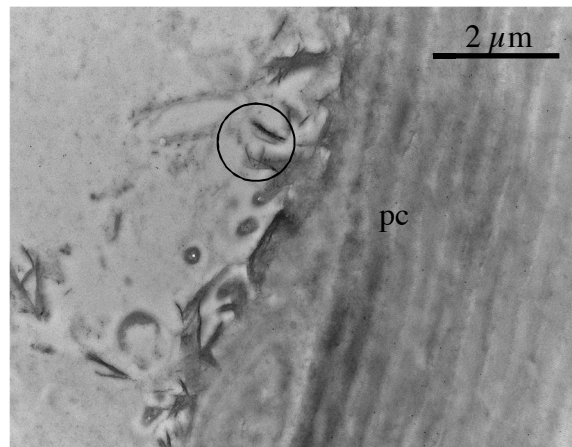


Fig.24: *Ibla cumingi* female: Label used was MAL II, Rabbit anti Biotin, 12nm gold. Procuticle (pc) not labeled. Cement (circle).

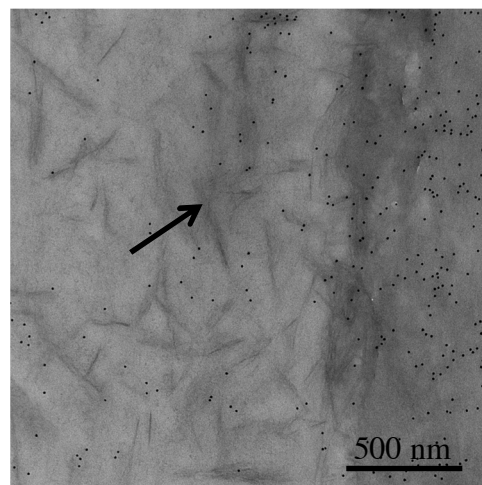


Fig.25: Detail of the cement bundles (arrow). WGA stain.

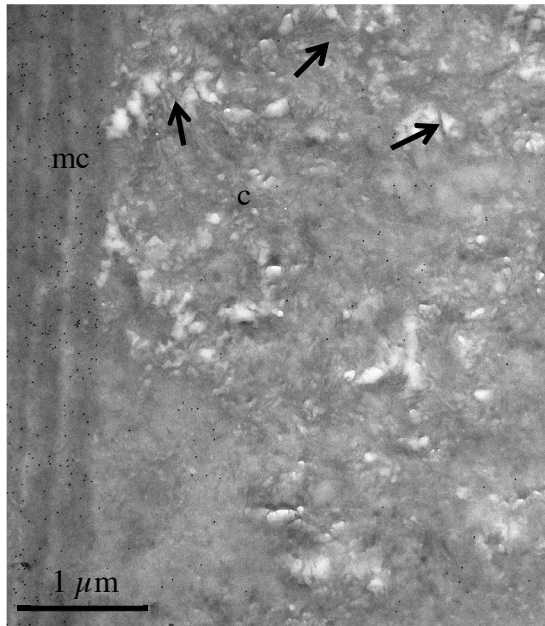


Fig.26: *Ibla cumingi* male: Body cuticle (mc) was labeled with WGA, Rabbit anti Biotin, 12nm gold. Cement (c) bundles (arrows)

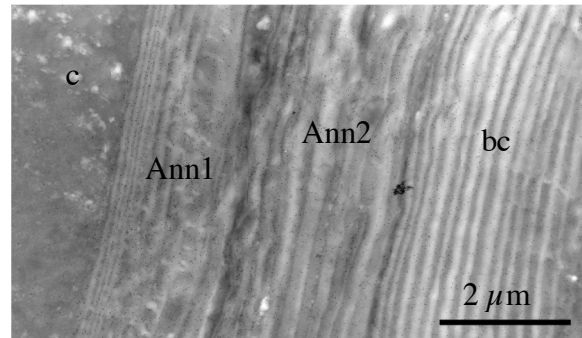


Fig.27: *Ibla cumingi* longitudinal section of the male: Antennules one and two (Ann1), (Ann2) Cement (c), and the body cuticle (bc). Labeled with WGA, Rabbit anti Biotin, 12nm gold.

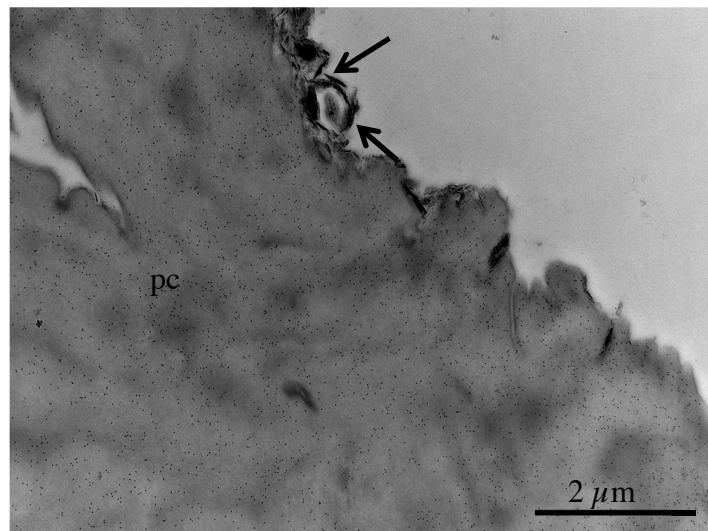


Fig.28: *Scalpellum scalpellum*: Procuticle (pc) labeled with WGA, Rabbit anti Biotin, 12nm gold, the arrows point to the cement.

Control

As control for the immunostain the primary antibody (WGA and MAL II) was omitted. The cuticle is nearly free of 12nm gold only gold particles not washed away stayed on creating slight background noise (Fig29,30).

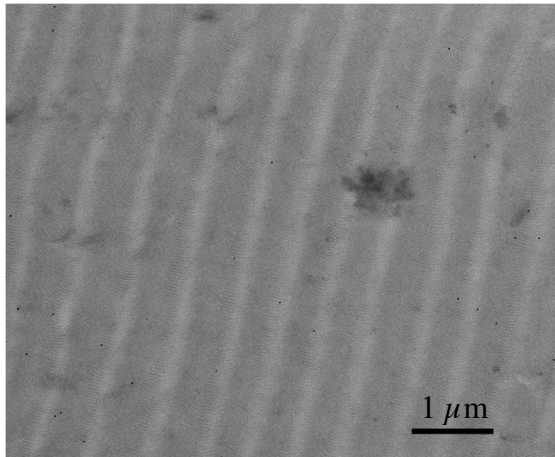


Fig.29: *Ibla cumingi* female: Attachment zone of the cuticle without primary antibody only secondary AB (Goat anti Rabbit) and 12nm gold.

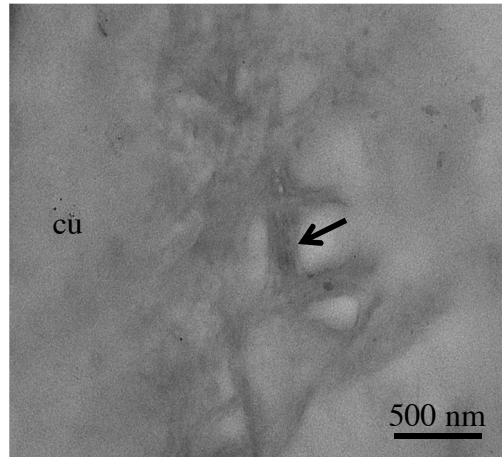


Fig.30: *Ibla cumingi* female: Cement bundles (arrow) and cuticle (cu), without primary AB.

Direct method

WGA was put directly together with 10nm gold on the sample. No intermediate step was used between them. The particle density on the cuticle appears lower than with an indirect labeled WGA lectin and no background noise is visible, there is a clear bond of the label and procuticle as with the other samples using WGA the epicuticle remains free of lectin due to the wax incorporation this area contains. The cuticle stained is from the peduncle near the attachment area showing a deeply lobed cuticle (Fig.31) and cement strands (Fig32).

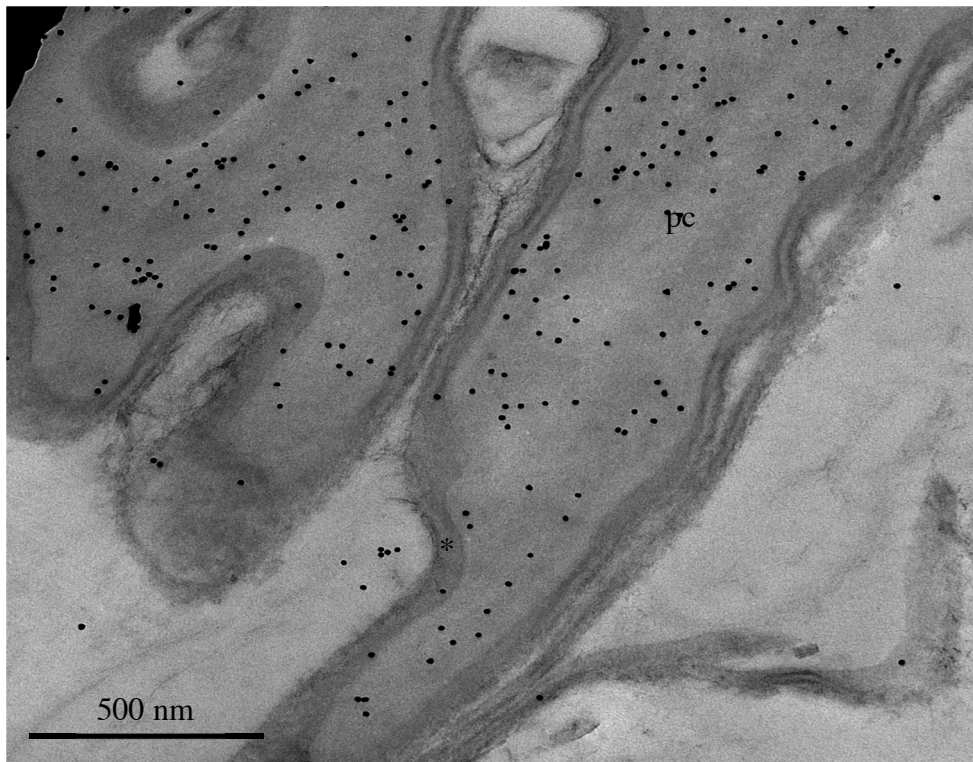


Fig.31: *Ibla cumingi* female. Procuticle (pc) and epicuticle (*) of the peduncle near the attachment zone. Structure of the cuticle looks lobulate. Stained with WGA+10nm gold.

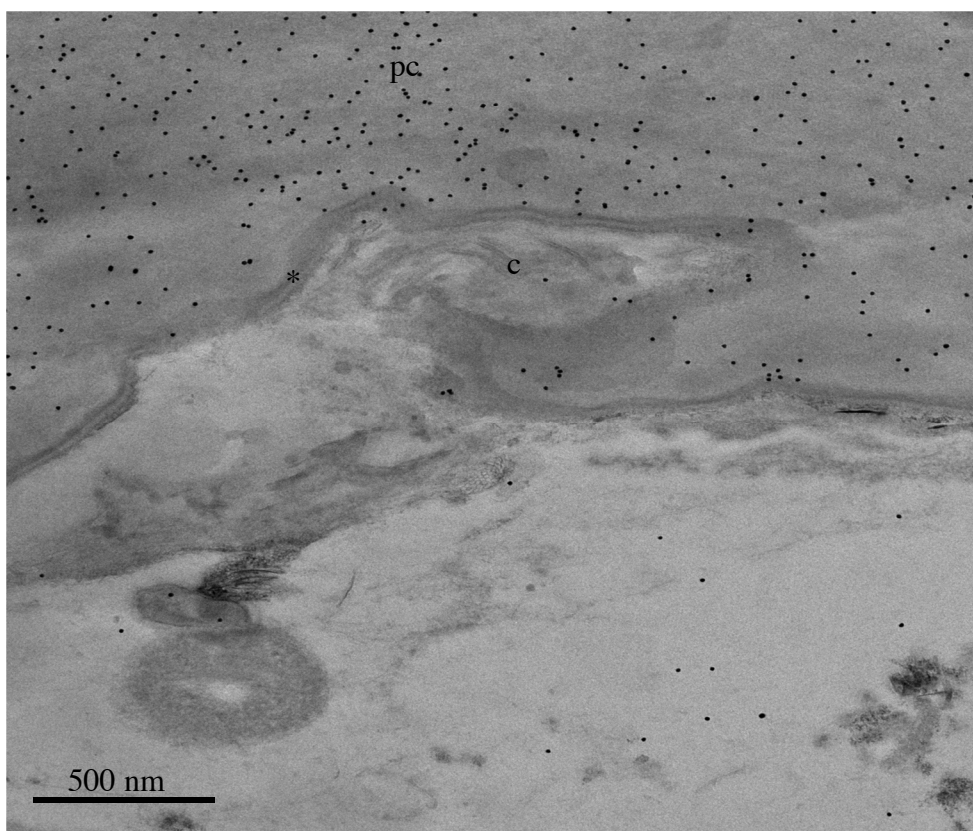


Fig.32: *Ibla cumingi* female. Procuticle (pc) stained with WGA+10 nm gold. Cement (c) around epicuticle (*)

4 Discussion

Measurements of the body cuticle of *Ibla cumingi* females reveal that this cuticle is much thinner than the cuticle of the peduncle. Given that, on the peduncle the cuticle serves as protection in the intertidal zone that was an expected result (Buckeridge & Reeves, 2009). In addition, measurements of the cuticle of the cirri were also taken. The cirri are motile for catching food and roil it into the mantle cavity for consumption (Klepal, 1985). As structures that can get extended outside of the mantle cavity where they are exposed to the strong currents of the intertidal zone, the cuticle of the cirri of the female *Ibla cumingi* is thicker than the body cuticle located inside the mantle cavity (Klepal, 1985). The cuticle of the cirri is also clearly distinguished into the layers, endocuticle, exocuticle and epicuticle. The cirri are innervated and muscled which is needed for the movement to catch prey and the rejection of unsuitable particles (Anderson, 1978, Gwilliam & Bradbury, 1971). Depending on their stage of development cirri can perform several types of activity such as normal beat, pumping, testing, fast beat and extension (Crisp & Southward, 1961). Even though the cirri of *Ibla cumingi* are from the serrulate type, which is one of the simpler types in which cirri can occur (Chan et.al, 2008), they can perform a movement which is called elongated extension (Chan et. al, 2008). The simple build is an indication for the ingestion of only small food particles (Klepal, 1985).

The cuticle of the peduncle of the female of *Ibla cumingi* varies in thickness. It is thickest at the basal end of the peduncle where the animal attaches to the substratum. There is also some excess growth of the cuticle forming lobes to better anchor the animal into the substratum, helping the animal with an additional physical aspect of attachment beside the chemical method of cement excretion. Following the way up to the opercular plates

the peduncle wall gets slightly thinner in the middle, and grows thicker again reaching the opercular plates. Concerning the distribution of the cuticular projections, at the basal part of the peduncle, there is the least amount of cuticular projections because this area of the peduncle is in contact with the substratum. The number of cuticular projections rapidly increases covering the rest of the peduncle up to the opercular plates. The opercular plates are free of cuticular projections.

Cement

Within the female mantle cavity males attach to the female by cement, which is secreted through the antennules of the male. Those antennules and a part of the male body following the antennules are partly embedded in the female tissue. The contracted female tissue, as reaction to the adherence of the male, can be seen on semithin sections in this work, Rodharth, E. (2013) and Buhl-Mortensen & Hoeg (2013). The male is not solely responsible for this attachment. Due to the bond that anchors the dwarf male to its place it is no longer able to shed the cuticle of the antennules resulting in the accumulation of concentric layers of cuticle during each molting cycle around them. Apart from that the male body cuticle is about twice as thick as the female body cuticle and lacks such concentric layers. Several studies show that the growth of barnacles on different substrata leads to different structured cement (Wiegemann & Watermann (2003) and (Berglin & Gatenholm, 2003). This also explains the differences between the cement of male and female in bundle thickness and quantity produced. The cement of *Ibla cumingi* differs not only between male and female but also from that of *Scalpellum scalpellum* (Rodharth, 2013). Also between species the cement differs in bundle thickness and quantity (Berglin & Gatenholm, 2003), (Zheden et al., 2012). Trying to differentiate cement from cuticle proved to

be difficult in some cases as the fibrous bundles within the cement matrix were not always identifiable using only observation of TEM. To distinguish between cuticle and cement the method of Immunogold labeling was applied. Thanks to the sugar-binding characteristics of wheat germ agglutinin (WGA) it was possible to bind this lectin to the sugar-containing cuticle leaving the proteinaceous cement free (Pesch, Riedel, & Behr, 2015). Immunostaining of the cuticle showed no results using the *Maackia amurensis* lectin (MAL II). MAL II binds to carbohydrate structures, which contain sialic acid (dos-Santos et al., 2014). Using WGA showed positive results as this lectin uses N-acetylglucosamine as receptor sugar (Chrispeels & Raikhel, 1991). The cuticle shows an alternating pattern where a dense layer of cuticular fibres which appears dark in TEM images follows a loose layer of cuticular fibres which appears lighter in TEM images. More gold particles were seen on the dense layer of the cuticle, as a result of more binding sites for the WGA. As WGA is preferential in binding to dimers and trimers of N-acetylglucosamine the darker layers seem to have them in higher abundance. There were no visible differences in the results of male and female *Ibla cumingi* cuticle using WGA. In both cases *Ibla cumingi* as well as in *Scalpellum scalpellum* WGA showed the same results with barely any background noise.

Cuticular Projections

Investigating the cuticular characteristics of *Ibla cumingi* special structures were found on the peduncle of the female. These structures were named cuticular projections. Unlike all other bristles studied by several authors so far like Beutel and Gorb (2001) or Chan et al., (2008), the cuticular projections on the peduncle of the *Ibla cumingi* female seem to be a characteristic only found in Iblidae so far termed as spines by Buckeridge &

Newman, (2006). They are consisting of packed cuticular substructures which are encompassed by a cuticular sheath forming a structure with no hollow inclusions. One epithelial cell forms one substructure. Growing densely on the peduncle these projections are directly in contact with the outside medium. Living in the intertidal zone *Ibla cumingi* settles in crevices and on other barnacles like *Tetraclita squamosa* (Bruguière, 1789) (Hoeg et al., 2009, Klepal, 1985).

Ibla cumingi at the base of the Thoracica is considered a primitive species (Anderson, 1994, Klepal, 1985). Its complex peduncular structures were compared with other rather primitive species like *Scalpellum scalpellum* and *Pollicipes pollicipes*. There are bristles on the peduncle of *Scalpellum scalpellum* but simpler in form than the cuticular projections of *Ibla cumingi*. Those structures found on the peduncle of *Scalpellum scalpellum* are hollow bristles grown from one cell. Bristles (chaetae) were studied by George & Southward (1973) and Orrhage (1973). The projections of *Ibla cumingi* have more in common with the bristles of annelids than with those of other barnacles (Westheide & Hermans, 1988). The structure of annelid bristles similar to the cuticular projections is also described by Gupta and Little (1970), George & Southward (1973) and Orrhage (1973). They describe these bristles as being composed of cylinders with walls of fine fibrils. Those bristles are formed by a single basal cell though which does not seem to be the case with cuticular projections of *Ibla cumingi* as there were rows of cells directly at the basal end of them to be seen. Although lacking the microtubules present in the bristles of polychaeta their setup is similar to those substructures described by Westheide & Hermans (1988). Westheide & Rieger (1988) showed a sketch of an annelid bristle and Southward et al (2005) published a TEM image depicting a girdle bristle of the polychaeta *Siboglinum atlanticum* Southward & Southward, (1958).

Scalpellum scalpellum and *Pollicipes pollicipes* have calcareous plates on their peduncle. But there are no structures indicating the presence of bristles on the peduncle of *Pollicipes pollicipes*. Like *Ibla cumingi* *Pollicipes pollicipes* is also distributed in the intertidal zone (Batham, 1945). Those species are living in tight spaces like nooks and crannies where they can be crowded with the danger of impeding each other. The cuticular projections may be used as “placeholder” to give the animal some space to have an uninterrupted flow of water rich with oxygen and feeding particles (Klepal, 1985). If the cuticular projections function as separators, the role of the cuticular projections in *Ibla cumingi* can be filled by calcareous plates in *Scalpellum scalpellum* and *Pollicipes pollicipes*. Sclerotized protuberances formed from single cells were termed as acanthae by Richards (1965) if they lack a socket and a sensory cell. As there were no sensory cells or other indications for sensory structures found in cuticular projections it can be assumed they have strict mechanical function, allowing the term acanthae also to be used for cuticular projections. Finding these cuticular projections only in Iblidae (Buckeridge & Newman, 2006) could mean that it is either a single gene characteristic (Dobzhansky, 1956) lost in higher species or the bristles found in *Scalpellum scalpellum* are a simplification of these projections.

Dwarf males retain a simpler developmental stage than their female (Klepal, 1985) or hermaphroditic counterpart (Pilsbry, 1908). For further study it would be advisable to investigate the bristles of the complementary dwarf males of *Scalpellum scalpellum*. The structure of the cuticular projections of *Ibla cumingi* females and the bristles on the cuticle of *Scalpellum scalpellum* dwarf males can be compared to get an indication if cuticular projections are an old characteristic already present in the

simpler males or if they were a secondary trait developed later only in Iblidae.

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6 Appendix

Acknowledgements

I want to thank Univ. Prof. Dr. Yair Achituv from the Bar Ilan University in Ramat Gan, Israel for the animals of the species *Ibla cumingi*. I Also want to thank Dr. Lene Buhl-Mortensen of the Institute of Marine Research in Norway for the *Scalpellum scalpellum* samples and Dr. Teresa Cruz from the Universidade de Évora, Portugal for the *Pollicipes Pollicipes* samples. I am very grateful to Dr. Heinz Schwarz for the antibodies, the nano gold and the knowledge to perform immunogold staining. Furthermore I want to thank Ao. Univ. Prof. Dr. Waltraud Klepal for her patience and knowledge to work with me and help me out wherever it was possible creating this script. Also a big thank you to Mag. Norbert Cyran for the help on the transmission electron microscope and to Mag. Daniela Gruber for teaching me the fixation of the animals. I also want to thank Vanessa Zheden PhD. and Johannes Suppan MSc. for helping me with the fixation of some of the animals.

Zusammenfassung

Ibla cumingi leben in der Gezeitenzone und sind somit starken Wasserbewegungen ausgesetzt. Wie andere Pedunculata benutzen sie proteinösen Zement um sich am Untergrund festzusetzen. Dieser Zement wird sowohl vom Weibchen für den Ansatz am Substrat als auch vom Männchen für die Anheftung an das Weibchen in deren Mantelhöhle benutzt. Da die Menge produzierten Zements derart gering ist wird angenommen, dass auch cuticuläre Strukturen an der Verankerung im Substrat beteiligt sind. Um die Beteiligung cuticulärer Strukturen zu untersuchen wurden die Regionen der Tiere, welche an der Festheftung beteiligt sind licht- und elektronenmikroskopisch untersucht. An der Basis des Peduncles des *Ibla cumingi* Weibchens fanden sich cuticuläre Auswüchse, die in die Spalten des Substrates hineinwachsen um besseren Halt zu bieten. Zusätzlich zur Anheftung mittels Zement in der Mantelhöhle des Weibchens findet in dessen Gewebe rund um die Antennuli des Männchens eine Reaktion statt die zu einer Kontraktion führt. In beiden Fällen lässt sich ableiten, dass die Festheftung sowohl chemischer als auch physikalischer Natur ist. Lobuläre Strukturen fanden sich auch an der Ansatzstelle des Peduncles des Hermaphroditen *Scalpellum scalpellum*, welcher ebenfalls zu einer Spezies sparsamer Zementproduzenten gehört. Immunogold Markierung der Cuticula erlaubte eine gute Differenzierung zwischen Cuticula und Zement.

Bei näherer Betrachtung des Stieles des Weibchens von *Ibla cumingi* fiel auf, dass die Borsten an dessen Stiel sich von anderen Borsten innerhalb der Crustacea unterscheiden. In dieser Arbeit wurde die Morphologie dieser cuticularen Anhänge genauer betrachtet. Es fiel auf, dass sie aus cuticulären Unterstrukturen bestehen welche von einem cuticulären Schaft umgeben sind. Im Gegensatz zu den üblichen Borsten der Crustacea sind sie

nicht hohl. *Scalpellum scalpellum* zeigte sowohl hohle Borsten auf dem Pedunkel als auch kalkhaltige Schuppen. Der Pedunkel von *Pollicipes pollicipes* zeigte nur kalkhaltige Schuppen.

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