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"Characterization of the cement of the stalked barnacle Dosima fascicularis (Crustacea, Cirripedia Thoracica): Morphology, biochemistry and mechanical properties"

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“If you can dream it, you can do it.”

Walt Disney
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I. Introduction

Barnacles (Cirripedia Thoracica) are sessile, marine Crustacea, which comprise acorn (Sessilia) and stalked barnacles (Pedunculata). As adult, all of them attach to the substratum with an adhesive (cement). An overview of the mechanism of adhesion was given in a preliminary study (Power et al. 2010). Barnacles have six free-swimming nauplius stages and one cypris larva. Prior to permanent settlement, the cyprid seeks a suitable place for attachment. During the exploration process (“walking” over surfaces with the antennules), it needs a temporary adhesive produced by unicellular antennular glands (Nott & Foster 1969; Phang et al. 2008). Once an appropriate site is selected, the larva secretes the permanent cyprid cement formed in paired cement glands within the cyprid carapace (Walker 1971; Phang et al. 2006). After metamorphosis to the subadult, cells of the cyprid cement glands first dedifferentiate and then contribute to the formation of the new cement apparatus. From now on the permanent cement of the adult is secreted (Walker 1973; Yule & Walker 1987). The cement produced in cement gland cells (in acorn barnacles located in the basal mantle, in stalked barnacles in the peduncle) is transported through a complex canal system. In many species this canal system consists of intracellular, collector, secondary and principal canals, in some species e.g. Balanus tintinnabulum and B. hameri the intracellular canal is lacking (Lacombe & Liguori 1969; Walker 1970). The cement is secreted to the outside through pores in the attachment disk on the third antennular podomere (Nott & Foster 1969). Cement production continues throughout life time of the animal in accordance with the moulting cycle (Fyhn & Costlow 1976; Kamino 2006). Most studies about cement synthesis were conducted on acorn barnacles. They secrete a thin, solid or reticulate layer of cement through a branching network of canals which lead to the outside with a number of pores at the base of the shell (Lacombe 1970; Walker 1973; Sangeetha et al. 2010). In contrast, pedunculate barnacles have the characteristic stalk (peduncle), an expansion of the forehead (Anderson 1994). The peduncle is attached to the substratum with the basal attachment disc of the
antennules (Lacombe & Liguori 1969; Walker 1974; Walker & Youngson 1975). From there a thicker cement layer is secreted than in the acorn barnacles.

Barnacles are among the most troublesome and dominant fouling organisms, because of their gregarious settlement on any hard substratum, whether it is natural or artificial (Knight-Jones & Crisp 1953; Wiegemann 2005; Kamino 2010, 2013). Accordingly, attention focused on fouling-release coatings, which prevent firm attachment of the barnacle cyprids and other encrusters (Schultz et al. 1999; Yebra et al. 2004; Aldred & Clare 2008; Scardino et al. 2008; Callow & Callow 2011). The adhesion strength of these organisms is reduced on low free energy surfaces, whereas higher free energy surfaces promote a much stronger bond between the attachment site and the surface (Yule & Walker 1987; Sangeetha & Kumar 2011). By using elastomeric coatings, the detachment stress can be lowered and barnacles or other encrusters can be removed easily from the substratum (Berglin & Gatenholm 1999; Kavanagh et al. 2003; Holm et al. 2009).

During the last decades the biochemical composition of the barnacle cement was in the focus of interest. The cement consists of approximately 90% proteins and is a multi-protein complex (Walker 1972; Kamino et al. 1996). More than ten cement proteins, of which five are unique among underwater adhesives, are identified in the Megabalanus rosa cement (Kamino 2006). Each of these proteins has a special characteristic and it is assumed that it fulfils either surface or bulk function in the underwater attachment process (Kamino 2010). The cement of barnacles is cured and remains durable underwater like the adhesives of other marine organisms e.g. mussels and tube-building polychaetes (Khandeparker & Anil 2007; Sangeetha & Kumar 2011; Stewart et al. 2011). There are, however, differences between the adhesives of these marine animals: The amino acid compositions and the primary structures of the barnacle cement proteins are highly complex and not as simple as those found in mussel byssal proteins and polychaete cement proteins (Kamino 2012). The adhesives of the mussels and polychaetes contain significant amounts of the amino acid L-3,4-dihydroxyphenyalanine.
(L-DOPA), a posttranslational modification of tyrosine, which plays an important role in interfacial adhesion (Nicklisch & Waite 2012; Wang & Stewart 2013). In contrast, no indication of L-DOPA (Jonker et al. 2012) nor of any other protein modification have been found in the holdfast system of barnacles with the exception of the glycosylated 52 kDa-protein found in the cement of *Megabalanus rosa* (Kamino et al. 2012).

Most pelagic stalked barnacles of the family Lepadidae are typical rafting organisms (Thiel & Gutow 2005) settling mainly on floating objects (Minchin 1996; Hinojosa et al. 2006). One of these species, *Dosima (Lepas) fascicularis* (Ellis and Solander, 1786), has developed a special floating device. It secretes a large amount of foam-like cement with gas-filled bubbles enclosed (Newman & Abbott 1980). The cypris larva of *D. fascicularis* attaches mainly on small, floating objects like feathers or parts of ruptured macroalgae (Cheng & Lewin 1976). After metamorphosis to the adult, the amount of cement increases and a voluminous cement ball may overgrow the substratum. The so formed float gives buoyancy to the animal and enables it to drift autonomously at the water surface (Thiel & Gutow 2005). Consequently, *D. fascicularis* is a cosmopolitan species, found in tropical and temperate seas, carried along by ocean currents (Young 1990).

Most studies about the buoy barnacle *Dosima fascicularis* refer to the ecology and distribution of this drifting animal and its substratum preferences (Cheng & Lewin 1976; Minchin 1996; Alvarez & Celis 2004; Hinojosa et al. 2006). The present thesis is an attempt to give an overall picture of the special adhesive of *D. fascicularis*.

Emphasis of the first manuscript (Zheden et al. 2012) is the morphology of the cement apparatus and the cement of this animal. The origin, formation and structure of the cement as well as its way through a complex canal system to the outside are presented. Also a hypothesis about the possible origin and the kind of gas inside the bubbles in the cement is
proposed. The methods used in this study are x-ray microtomography, light microscopy, transmission and scanning electron microscopy.

In our **second manuscript** (Zheden et al. 2014) interest focuses on the biochemistry of the *Dosima* cement, particularly on its protein and carbohydrate content. We analysed the amino acids and chemical elements. Our results are compared with the earlier investigations on the cement of *Dosima* made by Barnes and Blackstock (1974, 1976), of other barnacles (Kamino 2006; Barlow et al. 2010; Lin et al. 2014) and of the adhesives of other marine animals (Stewart et al. 2004; Flammang 2006; Silverman & Roberto 2007). The main methods applied in this study are scanning electron microscopy with energy dispersive X-ray microanalysis, 1D- and 2D-gel electrophoresis, mass spectrometry, FTIR- and Raman spectroscopy.

The **third manuscript** (Zheden et al. submitted-a) describes the mechanical properties of the cement of *Dosima fascicularis* considering differences between the outer and inner regions of the float. Using series of micro-indentations and tensile experiments, the hardness, the elastic modulus and the tensile stress of the cement float are investigated. These mechanical properties are compared with those of the rigid cement of acorn barnacles (Ramsay et al. 2008; Sullan et al. 2009; Sangeetha & Kumar 2011).

The **fourth manuscript** (Zheden et al. submitted-b) focuses on the buoyancy of this float. We adapted a force transducer apparatus for measuring the buoyant force of the cement. Further the water content, the gas volume inside the float and the correlation of the buoyancy and the gas volume inside the float are investigated. With our pressure experiments we could answer the question whether the foam-like cement float is an open or closed porous system.

**References**


II. Manuscript 1

Morphology of the cement apparatus and the cement of the buoy barnacle

*Dosima fascicularis* (Crustacea, Cirripedia, Thoracica, Lepadidae)


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Collection of material

Sample preparation for EM and LM

Cutting and staining of ultra- and semi-thin sections

EM and LM imaging and documentation

Performing statistical analyses

Manuscript preparation
Morphology of the Cement Apparatus and the Cement of the Buoy Barnacle *Dosima fascicularis* (Crustacea, Cirripedia, Thoracica, Lepadidae)

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Abstract. Barnacles produce a proteinaceous adhesive called cement to attach permanently to rocks or to other hard substrata. The stalked barnacle *Dosima fascicularis* is of special interest as it produces a large amount of foam-like cement that can be used as a float. The morphology of the cement apparatus and of the polymerized cement of this species is almost unknown. The current study aims at filling these gaps in our knowledge using light and electron microscopy as well as x-ray microtomography. The shape of the cement gland cells changes from round to oval during barnacle development. The cytoplasm of the gland cells, unlike that of some other barnacles, does not have distinct secretory and storage regions. The cement canals, which transport the cement from the gland cells to the base of the stalk, end at different positions in juvenile and mature animals. With increasing size of the cement float, the exit of the cement canals shift from the centrally positioned attachment disk of the vestigial antennules to more lateral positions on the stalk. The bubbles enclosed in the foam-like float are most likely filled with CO\textsubscript{2} that diffuses from the hemolymph into the cement canal system and from there into the cement.

Introduction

Many sedentary marine organisms such as mussels, tube-worms, and acorn and stalked barnacles attach to surfaces with proteinaceous underwater adhesives (Kamino, 2008; Stewart *et al.*, 2011). These substances have increasingly become a focus of interest in connection with research on antifouling (Schultz *et al.*, 2011) and on biomedical and biomaterial applications as potential biological adhesives (Khandeparker and Anil, 2007; Lee *et al.*, 2007; Ang *et al.*, 2011; Stewart, 2011; Yang *et al.*, 2011). Publication of comprehensive books on plant and animal adhesion (Smith and Callow, 2006; von Byern and Grunwald, 2010) further highlights the current interest in, and growing importance of, this field.

Barnacles (Cirripedia, Thoracica) are among the most troublesome and dominant fouling organisms due to their abundance and gregarious settlement (Knight-Jones and Crisp, 1953). Ships may lose up to 86% of their cruising speed because of heavy fouling (Schultz, 2007). Therefore, since the Second World War, attempts have been made to prevent the attachment of barnacles by developing resistant coatings for ships (Saroyan *et al.*, 1970b; Yebra *et al.*,...
The last larval stage, the free-swimming cyprid, settles and attaches to the substratum using the cement disk. The cement production continues in the adult during the growth period and in accordance with the molting cycle (Fyhn and Costlow, 1976; Walker, 1978; Kamino, 2006). This adhesive is produced in cement gland cells and conducted through a canal system to the area of attachment.


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The buoy barnacle *Dosima fascicularis* (Ellis and Solander, 1786) is unique among the Cirripedia (Thoracica) in producing a bulge of foam-like cement around the basal part of the peduncle by which it anchor the peduncle firmly in fissures of the substratum (pers. obs.). The buoy barnacle *Dosima fascicularis* is found on seaweed, feathers, seaweed, macroalgae (Boetius, 1952-53; Cheng and Lewin, 1976; Hinojosa et al., 2006), and inorganic material such as tar pellets and plastics (Minchin, 1996). As the barnacle grows, the substratum may become enclosed in the cement ball (Boetius, 1952–53), thereby allowing the animal to float independently just below the water surface.

Little is known about the cement apparatus in Pedunculata. In juvenile stalked barnacles the cement glands are at the apical region of the peduncle just beneath the capitulum. In adult individuals the glands may extend over most of the length of the peduncle, embedded between the ovaries (Lacombe and Liguori, 1969; Walker, 1974). The canal system leading from the cement glands to the base of the stalk can be differentiated by location and function into four canal sections: the intracellular, the collector, the secondary, and the principal (Lacombe and Liguori, 1969).

Little is known about the production and the structure of the highly unusual cement of *Dosima fascicularis*. Early biochemical analyses of the cement exist (Barnes and Blackstock, 1974, 1976), and a preliminary study to elucidate the mechanism of adhesion of this barnacle was carried out (Power et al., 2010). Our current interest is focused on the morphology of the cement apparatus and on the formation and morphological structure of the cement float of *D. fascicularis*. This knowledge is necessary for the understanding of the chemical and physical (mechanical) properties of the cement.

**Materials and Methods**

Juvenile specimens of *Dosima fascicularis* (Ellis and Solander, 1786) with a capitulum length of 2 to 6 mm and a width of 1 to 3 mm were collected on the western coast of Ireland. Adult, mature animals with a capitulum length of 10 to 25 mm and a width of 8 to 19 mm were collected on the northwestern coast of Denmark and on the western coast of Ireland. Only animals that had been washed ashore were collected. The peduncles of 5 juvenile and 10 mature animals were cut into small pieces, fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide, both at pH 7.3 in 0.1 mol l\(^{-1}\) sodium cacodylate buffer with 10% (w/v) sucrose, for 2 h. The samples were dehydrated in a graded ethanol series, and acetonitrile was used as intermediate before embedding in Agar low viscosity resin. All these steps were carried out at room temperature. Sections of 60 nm were cut on a Leica Ultracut-S microtome and stained with 0.5% uranyl acetate and 2% lead citrate. The sections were viewed in a Zeiss EM 902 transmission electron microscope (80 kV). Semithin sections (1 µm) were stained with toluidine blue and examined with a Nikon Eclipse E800 light microscope.

For scanning electron microscopy (SEM), the polymerized cement of adult specimens of *D. fascicularis* was fixed in 2.5% glutaraldehyde in 0.1 mol l\(^{-1}\) sodium cacodylate buffer and rinsed with distilled water. The wet cement was examined in a Philips XL 30 environmental scanning electron microscope (ESEM) (30 kV). The air-dried cement was coated with gold by an Agar B7340 sputter coater and examined in a Philips XL 20 scanning electron microscope (15 kV).

For x-ray microtomography (µCT), the glutaraldehyde-fixed stalk of a mature animal with the attached cement was rinsed in 0.1 mol l\(^{-1}\) sodium cacodylate buffer and dehydrated in a graded series of ethanol. Subsequently, the sample was treated with a 70% alcoholic solution of 1% (w/v) phosphotungstic acid for one week and afterward rinsed with distilled water. The sample was imaged at low resolution for an overview (9.6-µm voxelsize) and at high resolution for a close-up (2.4-µm voxelsize) with an Xradia MicroXCT-200 scanner while immersed in distilled water. This system uses a 90-kV/8 W tungsten x-ray source, a
cooled 2 k × 2 k CCD camera, and switchable scintillator-objective lens units. The schemata were created in Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA) based on the virtual longitudinal sections obtained by μCT.

For statistical analysis, the structured-walk technique known as the Richardson plot (Mandelbrot, 1967) was used to compare the ruggedness of the nuclear membrane of the young (n = 10) and the mature (n = 10) cement gland cells. On sections, the circumference of the nucleus was measured along its margin by using rods of different length. The fractal dimension is determined by plotting the log of the nuclear margin versus the log of the rod length (see Table 1).

Results

General morphology of the peduncle

The peduncle of Dosima fascicularis (Fig. 1A) tapers toward the base and becomes embedded in the cement as the float increases in size. The peduncle is covered by unarmored cuticle with an average thickness of 40 μm in juveniles and 100 μm in adults. The external thin epicuticle is 52 nm ± 31 (n = 20) thick. This and all confidence intervals represent standard deviations (SD). Within the internal procuticle, layers of electron-dense and electron-lucent material alternate. Exo- and endocuticle cannot be distinguished. The prismatic hypodermal cells (Fig.1B) underlying the
cuticle are 9.9 μm ± 1.9 (n = 60) high, and their oval nucleus is located in the center of the cell. Beneath the hypodermis is an outer layer of circular and a middle layer of oblique muscles, followed by the innermost layer with three to four rows of longitudinal muscles (Fig. 1C). A pair of antennulary nerves passes laterally through the peduncle. The wide hemolymph canal extends from the rostral sinus of the capitulum into the base of the peduncle, and its thin wall is formed by serially orientated connective tissue cells (Fig. 1D, E, Inset). Unicellular cement glands are in the upper part of the peduncle beneath the capitulum. In juvenile animals, two sets of cement glands are embedded in the reticulate connective tissue at the periphery of the peduncle. From there they extend toward the center of the peduncle (Fig. 2A). In mature animals, ovaries fill almost the whole peduncle and the cement glands are intermingled with them (Fig. 2B). A canal system leads from the cement glands to the base of the peduncle where the cement is extruded and hardens upon contact with the substratum and the seawater. Different sections of the canal system can be distinguished mainly by the appearance of the apical surface of their wall cells (see below).

Young cement glands

Young cement gland cells (Fig. 3A) occur only in small, juvenile animals. The cells are initially round, then acquire an oval shape. They are then 22.2 μm ± 3.1 long and 16.6 μm ± 3.3 (n = 30) wide. Around these cells is a thin layer of extracellular matrix (0.1 μm ± 0.02 wide, n = 20). The initially globular nucleus (fractal dimension D = 1.06, Table 1) is 10.7 μm ± 2.7 (n = 20) in diameter and rich in euchromatin. In the nuclear membrane are a high number of pores (13 ± 2 pores per 10 μm of membrane length, n = 10) (Fig. 3B). One electron-dense globular nucleolus is located centrally in the nucleus, measuring on average 5 μm in diameter. In very young animals the nucleolus has a sharply contoured interface with the nucleoplasm. The cytoplasm is full of vesicular rough endoplasmic reticulum (RER) and free ribosomes (Fig. 3B). Other organelles are few: Golgi bodies (consisting of two to three cisternae only), mitochondria, and vesicles (mostly lysosomes and multivesicular bodies). Some vesicles enclose membrane fragments (Fig. 3C). In young cement gland cells the intracellular canal has begun to form. The lumen of this canal is

Figure 2. Illustration of longitudinal section through the peduncle and the cement float. (A) In juvenile animals, young cement gland cells are situated close to the longitudinal muscles and connected to the canal system. The branched ends of the principal canals lead to pores at the attachment disk of the vestigial antennules. (B) In adult animals, mature cement gland cells are intermingled with the ovaries. The branched ends of the principal canals lead to lateral pores on the peduncle. Distally to the latter, previously used pores are visible in the cuticle of the peduncle. These pores are covered by layers of cement and have no connection to the cement canals. A large amount of cement is formed. a = alga, arrowhead = indicated position of hemolymph canal, cl = cement layers, cs = cement canal system, cu = cuticle of the peduncle, m = different muscle layers, mgc = mature cement gland cells, nc = newly secreted cement, o = ovaries, p = pores, pp = pores used previously, ygc = young cement gland cells. Scale bars: A, B = 500 μm.
usually very narrow, and few cell processes (0.08 μm in diameter) project into it. The gland cell is connected to the cells of the collector canal (Fig. 3C) and thus to the extracellular canal system.

**Mature cement glands**

Mature cement gland cells occur in animals that are in the reproductive stage. The gland cells are large, ovoid, 50 to 140 μm long and 40 to 50 μm (n = 35) wide (Fig. 4A). The layer of extracellular matrix around these cells is 0.21 μm ± 0.16 (n = 30) wide. Unlike the continuous smooth surface of the young gland cell, the surface of the mature gland has some narrow indentations, 0.08 μm ± 0.02 (n = 30) wide (Fig. 4B). The large oblong nucleus (37.2 μm ± 9.9 long, n = 20) of the mature gland cell is lobed (fractal dimension D = 1.42, Table 1, Fig. 4A) and contains up to 20 nucleoli embedded in heterochromatin. The nuclear membrane has 11 ± 1 pores per 10 μm of membrane length (n = 10). RER, free ribosomes, mitochondria, and Golgi bodies are distributed throughout the cytoplasm of the cell. The cisternae of the RER are round to oval (with a diameter of 0.13 μm ± 0.03, n = 40), or elongate (0.09 μm ± 0.02 wide, n = 40) (Fig. 4C). The elongate cisternae are often arranged parallel to each other, forming stacks (Fig. 4D). The Golgi bodies consist, on average, of three cisternae. Depending on the plane of the section, the shape of the Golgi bodies differs greatly: some are flat, others have a C- or S-shape, and some are almost round. On the trans-side of the Golgi bodies are

![Figure 3](http://example.com/figure3.png)

**Table 1**

*Comparison of the ruggedness of the nuclear membrane of the young and the mature cement gland cells with Richardson Plot (Mandelbrot, 1967)*

<table>
<thead>
<tr>
<th>Nuclear membrane</th>
<th>Juvenile animals</th>
<th>Mature animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (gland cells) n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean value of fractal dimension D</td>
<td>1.06</td>
<td>1.42</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.03</td>
<td>0.17</td>
</tr>
</tbody>
</table>
round vesicles (diameter from 0.2 to 0.5 μm) with fuzzy contents. Apart from these, many lysosomes and multi-vesicular and residual bodies are in the cytoplasm. In some such vesicles, crystals occupy up to a quarter of the vesicle volume (Fig. 4E). The intracellular canal with its lumen is prominent in mature gland cells. In the cytoplasm around the intracellular canal there is an especially high number of autophagic vacuoles (Fig. 4F). The cell processes, resembling microvilli (0.08 μm in diameter and 0.45 μm long), project into the lumen and are sometimes branched (Fig. 5A). Several vesicles (0.3 to 0.6 μm in diameter) with fuzzy content are found within the lumen. The intracellular canal is connected to the extracellular cement canal system.

**Extracellular canal system**

Depending on the distension of the peduncle, the cells that constitute the monoepithelial wall of the extracellular canal sections (collector, secondary, and principal canals) appear cuboidal or elongate, forming a squamous epithelium. Their round-to-oval nuclei lie mostly in the center of the cells. Like the gland cells, the canal is surrounded by an extracellular matrix (in the latter, 0.25 μm ± 0.11 wide, n = 30). The canal sections could not be distinguished by their epithelial height in adult animals (4 μm ± 1.8, n = 30). Slight differences could be observed between the collector canal and the other two canal sections in juvenile animals (collector canal: 4.3 μm ± 1.3, n = 20; secondary and principal canals: 6.1 μm ± 2.7, n = 40). The cells within

**Figure 4.** Transmission electron micrographs of the cement apparatus of a mature adult of *Dosima fascicularis*. (A) Overview of a mature cement gland cell with prominent intracellular canal and cross section of collector canal. The gland cell has a lobed nucleus with several nucleoli. The cell and the collector canal are surrounded by extracellular matrix. (B) Indentations of the gland cell membrane. (C) Elongate cisternae of the rough endoplasmic reticulum. (D) Golgi bodies and stacks of rough endoplasmic reticulum are present in the cytoplasm. (E) Vesicle containing crystals. (F) Autophagic vacuoles are in the cytoplasm in proximity to the intracellular canal (cut several times in the section). av = autophagic vacuoles, cc = collector canal, cw = collector canal wall, cy = crystals, ex = extracellular matrix, G = Golgi body, gc = cement gland cell, i = indentations, ic = intracellular canal, n = nucleus, nu = nucleoli, RER = rough endoplasmic reticulum. Scale bars: A = 10 μm; B = 1 μm; C = 200 nm; D = 1 μm; E = 200 nm; F = 1 μm.
one canal section and between two consecutive sections are connected by an adhesion complex, parts of which are septate junctions (Fig. 5A, Inset).

The collector canal, the first section of the extracellular system, is continuous with the intracellular canal of the gland cell and the secondary canal. Its wall consists of three to five cells. In juvenile animals, the lumen of the canal sections is at times very narrow and sometimes not even visible. A few vesicles with fuzzy contents are present in the lumen of the collector canal of mature animals (Fig. 5B). These vesicles are comparable to those within the gland cell and in the intracellular canal. The apical surface of the collector canal cells is almost smooth (Fig. 5C), apart from a few microvilli-like cell processes that have the same dimension as those in the intracellular canal. The collector canals of several gland cells lead to a single secondary canal, which in turn ends in two principal canals. In cross section, a single layer of three to five cells forms the wall of the secondary canal, as in the collector canal. The wall surface facing the lumen of the secondary canal is smooth. The principal canals are opposite to each other laterally at the base of the peduncle, close to the longitudinal muscles. They extend over two-thirds of the peduncle length. In juvenile animals they end in pores between the villi of the vestigial antennules' attachment disk (Fig. 6A). As the animal grows and the float enlarges, the antennules are lost and the principal canals branch laterally at the base of the stalk. The branches of the principal canal lead through the muscle layers and form new pores through the cuticle (Fig. 6B, Inset, C). When these are covered by the cement layer secreted last, new pores are opened more proximally on the stalk. The lumen of the principal canals is lined with a dense, probably chitinous, cuticle without any structure. The cuticle stains strongly with toluidine blue (Fig. 6C). In juvenile animals this cuticle is 0.18 μm ± 0.04 (n = 40); in mature animals it is 0.84 μm ± 0.22 (n = 40) thick. Within the lumen are round vesicles (Fig. 6C), electron-dense amorphous traces, or both, probably cement (Fig. 6D).

Cement

The polymerized cement usually forms an oval, whitish-beige foam-like float. Animals washed ashore carry bacteria, diatoms, and sand grains on the surface of the cement ball. Bacteria can also be found inside the cement float and in some bubbles. Small, cement-covered flies were found in the outer region of the float.

Young animals possess only a small amount of cement that hardly extends beyond the diameter of the peduncle. The adult animals (with a peduncle diameter of 4 to 5 mm at its basal end) have cement floats that are mostly oval with diameters between 8 and 15 mm. The polymerized cement
of animals attached to a large, smooth, and stiff surface (e.g., a plastic box) is flat and only about 5-mm thick. Its volume is about 1000 mm$^3$. In animals attached to small and soft surfaces (e.g., algae), the cement is globular and often surrounds the substratum completely. In these cases, the cement float is 7- to 10-mm thick and its volume is up to 2000 mm$^3$. The foam-like cement contains bubbles of different sizes (Fig. 7A). These bubbles are gas-filled and confer buoyancy to the animal. The cement float appears firm, but it is still elastic after fixation. The float consists of layers arranged concentrically around the stalk and the object to which it is attached. The layers are secreted from pores in the cuticle of the stalk, which are between 0.3- and 0.6-mm apart in mature animals. This distance corresponds to the thickness of the layers within the polymerized cement. Observed under the stereo microscope in wet conditions, the surface of the cement float appears gelatinous without any structure (Fig. 7A). Under the scanning electron
microscope the layers near the surface of the float are narrower than in the center, giving the appearance of a rind (Fig. 7B). Within this region bubbles are closely packed, rounded, and very small (11.36 μm ± 2.93 in diameter, n = 50). Some elongate bubbles, compressed in parallel to the surface of the float, are also present. In the cement around the stalk are many small rounded bubbles (25.42 μm ± 7.65 in diameter, n = 20). Next to these are large and elongate bubbles (470 μm to 2460 μm in length) with widened distal ends radiating to the periphery (Fig. 7C). The bubbles have a reticulated wall (Fig. 7D). In ultrathin sections the cement appears fibrous, sometimes forming condensed zones (Fig. 7E) that are often found framing the bubbles (Fig. 7F).
Discussion

The morphology of the cement apparatus of *Dosima fascicularis* is similar to that of *Lepas anatifera* (Lacombe and Liguori, 1969; Jonker et al., 2012). Both species belong to the family Lepadidae, whose members are early diverging barnacles considered to have retained ancestral features (Newman, 1982, 1987; Walker, 1992; Kugele and Yule, 2000). One of these ancestral features may be the absence of differentiation within the glandular cells—that is, the presence of large unicellular cement gland cells with uniform distribution of organelles in the cytoplasm (Lacombe and Liguori, 1969). In the more derived acorn barnacles (e.g., *Balanus tintinnabulum*, *Elminius modestus*, and *Chelonibia testudinaria*), a secretory (synthesis) and a storage (aggregation) region can be distinguished in the cytoplasm of the gland cell (Lacombe and Liguori, 1969; Lacombe, 1970; Walker, 1970, 1978). Another indication that *D. fascicularis* retains ancestral character states is that the maturation of the gland cells proceeds with that of the animal, as in the primitive balanid *Semibalanus balanoides* (Lacombe, 1970). It has been suggested that maturation of the animal and of the cement gland cells is synchronized with the molting cycle in the primitive, but not in the derived, barnacles (e.g., *Balanus psittacus*) (Lacombe, 1970). In the latter and in *Lepas anatifera* (Lacombe and Liguori, 1969), all stages of gland cell development can be observed in one animal at the same time. A more recent study (Jonker et al., 2012) has not shown this in *L. anatifera*, nor does it apply to *Dosima fascicularis*. Juvenile animals have only young cement cells; mature individuals only mature cells.

During maturation, the gland cells and their nucleus enlarge, the cells change their shape, and the number of nucleoli increases. The change of appearance of the gland cells during their development was also documented for *Lepas anatifera*, *Balanus nubilis*, *B. kondakovi*, and *Semibalanus balanoides* (Lacombe and Liguori, 1969; Lacombe, 1970; Karande and Gaonkar, 1977). The great number of nucleoli is a hallmark for highly active cells. In addition to the cement gland cells of barnacles, other gland cells are known to possess nuclei with many nucleoli (Lommelen et al., 2002), as also do oocytes in, for example, amphibians and fish (Shankar and Kulkarni, 2006; Mali and Bulog, 2010).

The cement gland cells of *D. fascicularis* are specialized for the synthesis and secretion of the mainly proteinaceous cement and are thus very active (Barnes and Blackstock, 1974, 1976; Walker and Youngson, 1975). The high activity of the cement gland cells is also indicated by the presence of a great number of nuclear pores, the numerous well-developed Golgi bodies, many free ribosomes, and the appearance of the rough endoplasmic reticulum (RER). Although the dimensions of the nuclei in young and mature glands differ greatly, the number of nuclear pores along a defined line segment is almost the same. The nucleus of the mature gland cell is up to 4 times bigger than the rounded nucleus of the young glands, and its surface is considerably larger due to its elongate and highly lobed form. In the young gland cells of *D. fascicularis*, the RER is vesicular and appears swollen. Swollen RER was also described in the cement gland cells of the cyprid of *Semibalanus balanoides* (Walker, 1973), in the cement cells of adult *Balanus haneri* and *Chelonibia testudinaria*, and especially in the synthesis region of *Elminius modestus* (Walker, 1970, 1978). This is generally interpreted as a sign of synthetic activity (Han and Bordereau, 1982), but it is also known in the context of programmed cell death (Klepal et al., 2008). In our samples of *D. fascicularis*, it cannot be ruled out that swelling of the RER (and of some mitochondria) could be due to the suboptimal condition of the animals, which had been washed ashore. In the mature cement glands are many residual bodies, some with crystalline inclusions. Like *Chelonibia testudinaria* and *Octolasmis mulleri*, *D. fascicularis* produces a large volume of cement, and residual bodies are a result of the high synthetic activity of the gland cells (Walker, 1978). The high production of proteins or the surplus of unused proteins may lead to the accumulation of these proteins as crystals in the residual bodies of *D. fascicularis*.

The formation of the intracellular canal in cement glands is only possible via the partial degeneration of the cytoplasm and some organelles. This explains the many autophagic vacuoles, lysosomes, and multivesicular bodies near the intracellular canal.

The cement gland cells release cement either by apocrine secretion as in *Lepas anatifera* (Lacombe and Liguori, 1969; Jonker et al., 2012) or as vesicles by exocytosis as in many acorn barnacles, for example, in *Balanus improvisus* or *Megalobalanus rosa* (Okano et al., 1996; Ödling et al., 2006). In our samples of *D. fascicularis*, the mode of secretion into the intracellular canal could not be observed.

It has been reported that the cement glands arise by differentiation of the wall cells of the secondary canal and that the cement canals originate from the invagination of the hypodermal cells of the exterior mantle wall (Lacombe and Liguori, 1969; Lacombe, 1970). Other authors suggest that the cement cells are derived from the collecting canal cells in the cyprid (Cheung and Nigrelli, 1975). So far we cannot confirm either suggestion about the origin of the cement gland cells because the available animals had developed too far to detect these processes. For such analyses newly settled cyprids and stages of their metamorphosis to the juvenile are necessary, which is made difficult by the fact that the lepadid barnacle *D. fascicularis* is a fast-growing species (Anderson, 1994).

Intracellular, collector, secondary, and principal canals are distinguished mainly by the appearance of the apical surface of their wall cells. In *D. fascicularis* the cell pro-
cesses projecting into the lumen decrease in number from the intracellular canal to the secondary canal. The principal canal is lined by a structureless chitinous-like cuticle as described for other barnacles (Lacombe and Liguori, 1969; Walker, 1970, 1974). In juvenile animals the principal canal leads into the attachment disk of the vestigial antennules, where the cement is extruded through several pores. As the animal grows the antennules are lost and new pores are formed through the cuticle at the distal part of the peduncle. From there the newly secreted cement is released, forming a new layer around the existing cement float. The new layer covers the pores that have been used for the previous cement excretion. When growth is continued, again new pores break through the cuticle more proximally on the stalk, and the next layer of cement is formed on top of the previous one. In this way the peduncle and its pores get more and more embedded in the cement float as the latter increases in size. The formation of new pores along with proceeding growth is also known in other Cirripedia Thoreacica, for example, **Semibalanus balanoides**, **Balanus crenatus**, and **Lepas anatifera** (Bocquet-Védrine, 1970; Walker, 1973; Kugele and Yule, 2000). In connection with active relocation, new pores are formed in the Pedunculata Capitulum mitella and *Pollicipes pollicipes*. In these species the pores and canals of the trailing edge are lost through sloughing, and their antennules cannot be detected anymore (Kugele and Yule, 1993, 2000). In the parasitic barnacle **Clistosaccus paguri** (Rhizocephalia), pores supposedly develop by chemical dissolution of the host and parasite cuticles around the distal end of the cyprid antennules (Høeg, 1985, 1990). The authors suggested that their cement contains lytic enzymes. It is possible that such enzymes are together with the cement also present in the principal canal of **D. fascicularis**. They could penetrate the cuticle of the stalk to form the pores.

The mechanism of extrusion of the cement differs in the various species of Cirripedia Thoracica. A kind of flushing fluid as described for some Balanidae may keep the ducts clear of any cement residues (Saroyan et al., 1970a). The vesicles seen in the principal canal of **D. fascicularis** may contain such a fluid. Cement secretion is confined to the short intermolt period (Walker, 1971, 1978; Fyhn and Costlow, 1976; Khandeparker and Anil, 2007), suggesting that cement synthesis and accumulation in the gland cell occur around the postecdysial stage. As the animal grows, the marginal area of the attachment region enlarges and must be continually fixed to the substratum (Kamino, 2006). The extrusion of cement in **D. fascicularis** is hypothesized to be regulated by hemolymph pressure (Power et al., 2010). In **Lepas anatifera**, oblique muscles between the cement cells aid the extrusion of secretion from the cement cells (Walker, 1974). In acorn barnacles, contraction of the striated basal muscles of the mantle layer is supposed to have the same function (Lacombe, 1970).

The cement in the principal canal is structureless, but the cement float has a specific foam-like appearance (Boëtius, 1952–53). The nature of the gas inside the bubbles is not known, and it is still not clear how it penetrates into the cement. It is most likely that CO₂, a byproduct of metabolism, is transported by the hemolymph to the collector and the secondary canals, where it enters their lumen. Our preliminary observations indicate that the pH of the cement float is 3 to 4. It is known that in an acid environment (below pH 4) more than 90% of CO₂ is gaseous. Together with the cement, CO₂ would be transported from the collector and secondary canals through the principal canal to the outside. The lipid bilayer of cell membranes is permeable to CO₂ and proteins lower CO₂ permeability (Gutknecht et al., 1977; Missner et al., 2008). When the cement and CO₂ are transported through the principal canal, the penetration of CO₂ through the proteinaceous cuticular lining of the canal may be negligible. In addition, increased hemolymph pressure within the peduncle may aid CO₂ diffusion into the collector and the secondary canals, thus preventing its diffusion back into the peduncular plexus. An excretion of CO₂ through the cement canal system could not only cause the formation of the gas bubbles but also contribute to the pH regulation of the hemolymph.

Two forces act on the cement float in opposite directions: gravity, which exerts traction downward due to the weight of the animal; and buoyancy, which exerts a pressure upward that is mostly caused by the light gaseous CO₂. This is seen in the elongate outline of the large bubbles radiating from the peduncle (caused by traction) and in their widened distal ends (caused by pressure of CO₂). A relationship between the size of the cement float and the buoyancy of the substratum on which the cyprids of **D. fascicularis** settled could not be determined in the available adult animals. However, the shape of the cement float seems to correlate with the surface properties of the substratum.

The cement floats of **D. fascicularis** individuals that have been washed ashore often seem to be surrounded by a “gelatinous rind.” This is newly secreted cement of low viscosity so that it can be transported through the cement canal and through the cuticular pores, as described for **Lepas anatifera** (Walker and Youngson, 1975). The cement layers in this rind are narrower than in the center, which can be explained by the increase in size of the float. Under the assumption that the animal can secrete only a certain quantity of cement at a time, the amount of newly produced cement available is only enough for the formation of layers that become narrower toward the periphery of the float.

The mechanism by which the newly secreted cement is transported from the pores in the peduncle over the entire surface of the float against gravity seems to depend on the Archimedes force on the cement (which has a lower density than seawater, further lowered by the bubbles within it), and the adhesive power of the cement and the gas within it.
When cement is excreted from the pores on the peduncle just below the cement float, adhesive forces cause the newly formed cement to adhere to that formed previously. This may also be aided by the pressure of the surrounding water. The low density of the cement causes the animal to rise to the water surface. The light gaseous CO$_2$ within the cement may produce an additional uplift, allowing the newly formed cement to spread all over the surface of the float. Flies stuck to and partly embedded within the cement prove that the cement float of stranded individuals of *Doxima fascicularis* was still very soft and sticky on its surface. This could either mean that the *D. fascicularis* cement takes a relatively long time to harden or that cement secretion in this particular individual was going on while the animal was washed ashore.

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III. Manuscript 2

Biochemical analyses of the cement float of the goose barnacle

*Dosima fascicularis* - a preliminary study

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Sample preparation for and performance of 1D- and 2D-gel electrophoresis

Manuscript preparation
Biochemical analyses of the cement float of the goose barnacle *Dosima fascicularis* – a preliminary study

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The goose barnacle *Dosima fascicularis* produces an excessive amount of adhesive (cement), which has a double function, being used for attachment to various substrata and also as a float (buoy). This paper focuses on the chemical composition of the cement, which has a water content of 92%. Scanning electron microscopy with EDX was used to measure the organic elements C, O and N in the foam-like cement. Vibrational spectroscopy (FTIR, Raman) provided further information about the overall secondary structure, which tended towards a \(\beta\)-sheet. Disulphide bonds could not be detected by Raman spectroscopy. The cystine, methionine, histidine and tryptophan contents were each below 1% in the cement. Analyses of the cement revealed a protein content of 84% and a total carbohydrate content of 1.5% in the dry cement. The amino acid composition, 1D/2D-PAGE and MS/MS sequence analysis revealed a de novo set of peptides/proteins with low homologies with other proteins such as the barnacle cement proteins, largely with an acidic pI between 3.5 and 6.0. The biochemical composition of the cement of *D. fascicularis* is similar to that of other barnacles, but it shows interesting variations.

**Keywords:** barnacle adhesive/cement; 1D/2D PAGE; FTIR/Raman spectroscopy; protein primary/secondary structure; hydrogel; carbohydrate

**Introduction**

Proteinaceous underwater adhesives are secreted for locomotion by the sea star and sea urchin (Flammang et al. 1998; Santos et al. 2009), for defence by the sea cucumber (DeMoor et al. 2003), for building protective tubes by sabellariid polychaetes (Stewart et al. 2004) and for attachment to the substratum by mussels and barnacles (Kamino et al. 1996; Waite 2002). The best characterised marine bioadhesive systems are those of the sandcastle worm *Phragmatopoma californica* (Stewart et al. 2004), the blue mussel *Mytilus edulis* (Silverman & Roberto 2007) and the acorn barnacle *Megabalanus rosa* (Kamino 2008). The adhesives of the sandcastle worm (Wang et al. 2010) and mytilid mussels (Waite 2002) contain significant amounts of the amino acid L-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA can complex with metal ions and oxides (Sever et al. 2004), as well as with semimetals, such as silica, therefore allowing adherence to rocks and glass (Silverman & Roberto 2007). The holdfast system of the barnacle differs from that of mytilid mussels and sabellariid polychaetes by the lack of a detectable L-DOPA system and the slow curing process of the adhesive (Naldrett 1993; Kamino et al. 1996; Kamino 2010; Nicklisch & Waite 2012). Barnacle cement is produced by unicellular glands throughout the lifetime of the animal in accordance with its moult cycle (Saroyan et al. 1970; Fyhn & Costlow 1976; Jonker et al. 2012; Zheden et al. 2012). Two types of adhesive can be distinguished: the primary cement, produced while the animal is attached to a substratum, and the secondary cement, secreted when the animal is injured or detached (Saroyan et al. 1970, 1970; Kamino et al. 1996). The two kinds of cement are very similar in their amino acid composition (Naldrett 1993) and consist of ~90% protein (Walker 1972; Kamino et al. 1996). Usually the secondary cement is used for biochemical analyses, because it is easier to collect.

Most studies on the cement proteins of cirripedes have been conducted on acorn barnacles, especially *Megabalanus rosa*. Its cement is composed of more than 10 proteins, of which five are novel in their primary structure compared with other underwater adhesives (Kamino 2006). Each protein has a unique characteristic and it is assumed that it fulfils either a surface or bulk function in the multifunctional process of underwater attachment.

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(Kamino 2010). Only a few studies exist on the biochemical composition of the cement of the buoy barnacle Dosima (Lepas) fascicularis (Ellis & Solander 1786) were by Barnes and Blackstock (1974, 1976). They determined the carbohydrate, lipid and amino acid composition as well as the electrophoretic properties of the cement. Walker and Youngson (1975) focused on the related goose barnacle Lepas anatifera and assumed that the cement of acorn and goose barnacles had a similar biochemical composition, but that it may act in a different way. Goose barnacles normally secrete a thick layer of primary cement in contrast to the thin film produced by acorn barnacles (Walker & Youngson 1975). Most pelagic goose barnacles of the family Lepadidae are, like acorn barnacles, known to be major fouling organisms. They often cover floating objects such as ships, buoys, tar pellets, plastic, driftwood, feathers and seaweed (Boëtius 1952; Cheng & Lewin 1976; Minchin 1996; Hinojosa et al. 2006). Dosima fascicularis does not usually cover large surfaces. It clusters either on organic or inorganic substrata of different size or on the cement of conspecifics. Its stalk is flexible and the plates are fairly soft. It is unique among the barnacles due to the amount and morphological structure of the cement it produces. It secretes layer by layer an excess amount of foam-like cement (Zheden et al. 2012) (Figure 1 and Supplementary Figure S1). [Supplementary material is available via a multimedia link on the online article webpage.] The bubbles in the foam-like cement are probably filled with CO$_2$, a metabolic product of the haemolymph (Zheden et al. 2012). As the animal grows, small substrata such as feathers or pieces of seaweed can be surrounded and sometimes enclosed by the enlarging cement, which then forms a float (Figure S1a, b). As a result, D. fascicularis can drift on the surface of the water (Boëtius 1952–53; Young 1990).

In the present study some of the biochemical components of the cement of D. fascicularis were investigated. The large amount of primary cement is easily accessible and therefore ideal for analysis. Although there is overall agreement on the composition of the cement of D. fascicularis compared with that of other barnacles, some differences are noticeable, including the acidic composition of the adhesive proteins, their structural architecture, the absence of Raman detectable disulphide bonds and the very low amounts of cystines.

Materials and methods

Dosima fascicularis was collected during the summer months of 2012 and 2013 on the west coast of Denmark (56°57’37.63” N, 8°21’37.60” E) after having been washed ashore. Only primary cement was used for the analyses.

General cement analysis

The cement was removed from the animal and any adherent substratum. Pieces of cement were washed in distilled water to remove debris and salt before freezing at −20°C or freeze-drying (Christ Alpha1–4, Christ GmbH, Osterode, Germany). To estimate the water content of the cement, four samples of three different individuals of Dosima were weighed before and after freeze-drying. For the protein analyses, the freeze-dried samples were powdered in a small porcelain mortar.

Total carbohydrate analysis of the cement

Carbohydrate analysis was performed using a kit from BioVision (Catalogue # K645–100. Milpitas, CA, USA). This is based on the phenol-sulphuric acid method and can detect most forms of carbohydrates, including simple and complex saccharides, glycans, glycoproteins and glycolipids. The experiments were performed according to the supplier’s manual. Freeze-dried cement and glucose for the standard curves (supplied in the kit) were completely dissolved in 150 µl of concentrated H$_2$SO$_4$ (98%) and incubated at 90°C for 15 min. Thereafter 30 µl of developer were added and the mixture was mixed on a shaker for 5 min. Control analysis took place without the developer. The samples were detected spectrophotometrically at 490 nm with a multimode reader Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany). The total carbohydrate content of the freeze-dried material was calculated on the basis of the glucose standard curve via linear regression analysis.

Staining methods

The periodic acid-Schiff (PAS) method (McManus & Mowry 1960) was used to detect the presence of neutral hexose sugar units in the cement. The negative control did not contain periodic acid. The slime of the slug Arion rufus served as a positive control. To test for the possible presence of L-DOPA in the cement, pieces were stained according to the protocol of Arnow (1937). Samples from the tube-dwelling polychaete Sabellaria alveolata were used as a positive control (Becker et al. 2012).

Scanning electron microscopy with energy dispersive X-ray microanalysis (EDX)

The cement float was cut into pieces, washed with distilled water overnight and air-dried. The elemental composition was determined without any carbon coating on the samples using a Philips XL 20 scanning electron microscope (SEM) (Eindhoven, The Netherlands) equipped with an energy dispersive X-ray spectrometer with a lithium-drifted silicon (SiLi) detector crystal.
Solubilisation experiments

The freeze-dried cement was solubilised in thiourea/urea lysis buffer (2 M thiourea, 7 M urea, 2% (w/v) CHAPS), 2% (w/v) and alternately 5% (w/v) dithiothreitol (DTT)) by incubation at 37°C overnight with stirring (Rabiloud 1998). Afterwards the samples were centrifuged at 21,000 x g for 15 min. The supernatant was removed and stored (−20°C). The remaining pellets were dissolved in SDS sample solubilisation buffer (1% SDS, 100 mM Tris-HCl pH 9.5) and incubated at 37°C for 3 h with stirring. After centrifugation the final soluble fraction was stored (−20°C).

Electrophoretic analyses

Samples for one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) were incubated at 95°C for 15 min in Laemmli sample buffer (Bio-Rad, Munich, Germany). The samples were run on a 1D precast 10–20% polyacrylamide ready gel (Bio-Rad) with two different amounts of protein (10 and 20 μg). Precision Plus Protein Dual Colour Standards (Bio-Rad) were used as markers. Gels were run at 100 V, 50 mA for 90 min in SDS PAGE running buffer 1x (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS at pH 8.3). The proteins were visualised by staining with Page Blue™, Protein Staining Solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). For the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the final soluble fraction was precipitated with 20% trichloroacetic acid (TCA) and 0.2% DTT in ice-cold acetone. IEF hydration solution (2 M thiourea, 6 M urea, 1% (w/v) CHAPS, 1% (w/v) DTT, 0.5% (w/v) IPG buffer pH 3–4) was added to the fractions and incubated at 37°C for 20 min with stirring followed by consecutive centrifugation at 21,000 x g for 5 min. The protein solution (~150 μg of protein) was applied to a ReadyStrip™ IPG Strip (pH 3–10, 11 cm) (Bio-Rad). Isoelectric focusing (IEF) was performed with an Etan IPGphor3 unit (GE Healthcare, Sweden) for 13,000 Vh at 50 μA strip−1. The strips were equilibrated in equilibration buffer 1 (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% (w/v) DTT) and equilibration buffer 2 (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 4% (w/v) iodoacetamide) with gentle agitation for 15 min each. 2D-PAGE for each strip was performed at 200 V, 50 mA for 50 min in precast 10–20% polyacrylamide ready gels for IPG strips (Bio-Rad). Proteins were visualised by overnight staining in Page Blue™ and destained in distilled water (Grunwald et al. 2007).

Sequence analysis

Gel slices from 1D-PAGE gels of protein bands at a size of 85.2, 68.4, 63.5 and 60.5 kDa were digested by trypsin/chymotrypsin according to the company’s protein digestion instructions for In-Gel samples (protocol and performance by Proteome Factory AG, Berlin, Germany). The acidified peptides were applied to nanoLC-ESI-MS (LTQ-FT, Thermo Finnigan) analyses using a 35 min nanoLC gradient (Agilent 1,100 nanoLC system) with solvent A (0.1% formic acid, 5% acetonitrile, 94.9% dH2O) and solvent B (0.1% formic acid, 99.9% acetonitrile). The mass accuracy was better than 5 ppm for MS data and ± 0.5 Da for MS/MS data. MS/MS was subjected to de novo sequencing. The induced modification propionamide adducts (at cysteine) and common sample handling modifications were allowed during the MS/MS data search. To minimise false positive hits, common laboratory proteins (ie trypsin, keratin) were taken into account and their peptide signals were disregarded. Each candidate sequence was used for a BLAST alignment search to further exclude contaminants.

The de novo sequencing of the MS/MS spectra was performed by using the PEAKS® program (Bioinformatics Solutions Inc., Waterloo, ON, Canada). This software
computed the best peptide sequences based on the fragment ions collected from the peaks in the MS/MS spectrum (Zhang et al. 2012). The SPIDER program (Han et al. 2005) was used in conjunction with the PEAKS tool to perform database searches for the MS/MS generated sequence tags. The SPIDER tool is able to cope with common sequence substitutions such as I/L, N/G, SA/T/M in the *de novo* generated sequence tags. The ALC score was used as selection parameter for the MS/MS sequence tags for the resulting Dosina peptide sequence (see Table 2); only the sequences with the highest score are given here. (De novo sequencing by MS/MS is less reliable than chemical methods such as the EDMAN degradation and therefore makes scoring of the tags necessary. The selection parameter for the candidates was their ALC score for the given sequence and only *de novo* peptide sequences with the very highest score were given.)

The identified peptide sequences were additionally analysed by a NCBI blastp search in non-redundant database. The blastp search algorithm automatically adjusted the parameter to find optimal matches to short peptides. The search was made first without limitations in the taxa settings, and second only with the search option barnacles (taxid:6,676). The first two results of each peptide were displayed and a selection of other identified sequences is shown in Table S1.

**FTIR and Raman spectroscopy**

Protein samples were measured as freeze-dried powder without any further procedure and (b) after dissolution in common D$_2$O (Sigma Aldrich, Munich, Germany). For D$_2$O exchange a fresh piece of cement was washed five times with ultra pure water (0.05 μS cm$^{-1}$) for 1 h. After this initial washing step, the cement piece was immersed in 2 ml of D$_2$O for 7 days (the D$_2$O solution was changed daily).

For interpretation of the results normal peak picking was used. The Fourier transform infrared (FTIR) measurements were performed on a Bruker Equinox 55 instrument (Bruker Optics GmbH, Ettlingen, Germany) with a Harrick Golden Gate ATR inlet (diamond crystal, one reflection). Measurements were taken with a resolution of 4 cm$^{-1}$ and 32 scans. Background measurements were taken against air. The FT-Raman measurements were performed on a Bruker Vertex 80 with RAM II module (Bruker Optics). A 1,064 nm Nd-Yg laser was used. Measurements were taken with 250 scans at a resolution of 4 cm$^{-1}$.

The spectra and images were evaluated using the Bruker software package OPUS 6.5 and the CONFOCHECK system (Bruker Optics), which is a dedicated FTIR system for the investigation of proteins. Identified peaks were aligned manually and plotted on the spectra.

**Results**

**SEM with EDX**

The excess amount of foam-like cement produced by *D. fascicularis* (Figure 1a, Figure S1) consisted of concentrically arranged layers with gas-filled bubbles of different sizes (Figure 1b, c). Near the surface of the cement, the layers were closer packed, giving the appearance of a rind containing small bubbles (Figure 1b, c).

EDX analyses gave a rough overview of the elemental composition of the primary cement. The elements carbon (C), oxygen (O) and nitrogen (N) had the highest counts in the spectrum, followed by sulphur (S) and magnesium (Mg). Calcium (Ca), phosphorus (P) and potassium (K) were present only in smaller quantities (Figure 2a). EDX line scan spectra and dot mappings of the elements showed that C, O and N were distributed evenly throughout the cement (Figure 2b, c). S, Mg, Ca, P and K seen in the spectra were not detected by line scans and dot mappings, supposedly because the amounts of these elements were below the detection limit of the system.

**Water, carbohydrate and amino acid analysis**

The cement had a high water content of 92% ± 2.7 (mean ± SD; n = 4), estimated by comparing the fresh and freeze-dried samples. The cement itself had the consistency of a stable gel, which could not be broken down by the fingers. The dried cement was found to contain 1.5% ± 0.08 (mean ± SD; n = 4) carbohydrates, as was confirmed by PAS staining (Figure S2a).

The amino acid analysis showed that the total protein content of the cement was at least 84%. Eighteen amino acids were identified by hydrolysis (Table 1). The amino acids AsX (asparagine or aspartic acid) and GIX (glutamine or glutamic acid) were most prominent, each at about 10% (Table 1). The methionine, histidine, cystine and tryptophane amino acids were only found in small quantities (<1%). The proteins contained slightly more polar (51%) than non-polar (49%) amino acids. High amounts of amino acids with hydrophobic side chains (about 10%) and only small amounts of basic amino acids (about 1%) were detected in the cement.

In summary, the cement was composed of (w/w) 92% water, 6.7% proteins (84% of dry cement), 0.12% carbohydrates (1.5% of dry cement) and 1% undefined substances. Part of the latter could be salts from the seawater or lipids.

**Solubility and electrophoretic properties**

The cement could not be fully solubilised with a thio-urea/urea lysis buffer and some unresolved debris remained. At least 10 proteins were identified using 1D- and 2D-PAGE stained with coomassie dye Page Blue™.
Their molecular mass ranged from 47.4 to 205.0 kDa (Figure 3a). The apparent molecular masses of the prominent proteins were 60.5, 63.5, 68.4, 85.2, 111.4, 149.3 and 205.0 kDa. The 2D-PAGE showed three additional protein bands the molecular mass of which could not be confidently determined due to the lack of marker bands above 250.0 kDa. All the detected proteins were acidic (or neutral) with an isoelectric point (pI) ranging from 3.5 to 6.0 (Figure 3b). The prominent proteins in the 2D gel had molecular masses of 47.4, 60.5, 63.5, 68.4, 135.4, 149.3 and 205.0 kDa. Following the nomenclature adopted by Kamino et al. (2000) the D. fascicularis cement proteins were named according to their molecular masses (Dfcp-60, -63, -68 and -85).

**Sequence analysis**

The sequence tags in the four different protein fragments obtained from the 1D-PAGE and following PEAKS (Zhang et al. 2012) and SPIDER search (Han et al. 2005) indicated a variety of possible proteins. Each of the identified 20 peptides (Table 2) was further analysed.
by NCBI blastp database search (Altschul et al. 1997).

In a first approach the peptides were searched against the non-redundant protein sequence (nr) entries without taxa limitations. Within these results, no conclusive assignment of the sequences of the bands with the molecular masses 60.5, 63.5, 68.4 or 85.2 kDa to a specific protein and/or to a related barnacle species was possible. In a second approach the search was limited to

Figure 2. SEM images and EDX spectra of the cement of D. fascicularis. (a) EDX spectrum of the elemental composition of the cement float (left). The mean values in weight per cent (Wt %) and the internal error (Error) are shown on the right side. (b) SEM image with the position of the line scan (left). The associated EDX line scan spectrum (right) shows high counts of carbon (C), oxygen (O) and nitrogen (N). Other elements such as sulphur (S) are not detected with this method. (c) SEM image (left) and corresponding dot mappings of the elements C, O, N and S (right). Only C, O and N give fair signals and an even distribution of the elements; S gives a random distribution of noise signals. Scale bar: b, c: 50 μm.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Goose barnacles</th>
<th>Acorn barnacles</th>
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<td><em>D. fascicularis</em></td>
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<tr>
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<tr>
<td>Protein content</td>
<td>84%</td>
<td>75.9%</td>
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Figure 3. 1D-PAGE and 2D-PAGE of the solubilised *D. fascicularis* cement proteins. (a) 1D-PAGE. Excised gel bands at 85.2 (Dfcp-85), 68.4 (Dfcp-68), 63.5 (Dfcp-63), 60.5 kDa (Dfcp-60) of the coomassie-stained gel after 1D-PAGE of the final soluble fraction of the cement of *D. fascicularis*. 10 μg and 20 μg of protein were applied to the 1D gel. (b) 2D-PAGE. Corresponding positions of the excised protein bands in the coomassie-stained gel after 2D-PAGE of the final soluble fraction after TCA acetone precipitation of the cement.
Table 2. *De novo* peptide sequences and PEAKS® results gained from sequence analysis of protein bands obtained from PAGE of the cement of *D. fascicularis* by MS/MS analysis.

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<th>m/z</th>
<th>z</th>
<th>ppm</th>
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<td>2.0</td>
<td>90 92 87 87 91 88 80 77 49</td>
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</table>

Dfcp: *D. fascicularis* cement protein (followed by the molecular mass). 'Local confidence is the confidence that a particular amino acid is present in the *de novo* peptide at a particular position. It is presented as a percentage. Total local confidence (TLC) is the sum of the local confidence scores (0 to 1) from each amino acid in the peptide sequence. Average local confidence (ALC) is the average of the TLC. It is TLC divided by the number of amino acids in the peptide sequence. ppm = precursor mass error, calculated as 10^6 × (precursor mass – peptide mass) / peptide mass. m/z = precursor mass-to-charge ratio. z = precursor charge.' Source: PEAKS® user manual (http://www.bioinfor.com/peaks/support).
the taxon barnacles. Table S1 shows the summarised results of over 2,000 hits. The first two identified proteins of each Dfcp peptide with the lowest E-value were displayed. Other results with higher E-values (= less reliable) are exemplarily shown to display possible homologue sequences in other barnacle proteins. It must be remembered that, due to the short peptide sequences, false positive results can occur.

Within the identified proteins the barnacle cement proteins cp-100, cp-20 and cp-19 were found, but never with the lowest E-value. In addition, the cement proteins were spread over the DfcpS like following: Dfcp-60 with 1x cp100; Dfcp-63 with 7x cp100 and 1x cp19; Dfcp-68 with 2x cp100, 1x cp20 and 1x cp19; Dfcp-85 with 4x cp100 and 1x cp19 matches. E-values <1 were found only for the Dfcp-60 (TPLSLES(VTR) matching ‘neurofibromin’, Dfcp-63 (WVTSAWSSKAR) matching ‘lectin BRA-2’, Dfcp-68 (FASEADLDDLTVK) matching ‘clathrin heavy chain’ and Dfcp-85 (RRGLVLSHLAQPK) matching ‘settlement inducing protein complex’ (Table S1). Furthermore, >20 times sequences from the barnacle protein ‘MULTIFUNCin’ were identified.

**FTIR spectroscopy**

The analysis showed significant peaks corresponding to proteins containing a β-sheet structure (amide I between 1,640 and 1,660 cm$^{-1}$) and/or amyloid-like structure (amide I between 1,630 and 1,610 cm$^{-1}$) (Barlow & Wahl 2012). Distinct peaks at 1,517 cm$^{-1}$ and 1,446 cm$^{-1}$ in the spectrum of the dried and D$_2$O equilibrated sample respectively indicated amide II vibration in agreement with the results of Omoike & Chorover (2004) and Barlow and Wahl (2012). The proteinaceous cement that was analysed did not contain detectable amounts of phosphate (1.253 cm$^{-1}$) (Gremlich & Yan 2001). Multiple peaks between 1,236 cm$^{-1}$ and 950 cm$^{-1}$ corresponded to amide III vibration (1,234 cm$^{-1}$) (Cai & Singh 2004). In addition, vibrations of polysaccharides (1,150–1,000 cm$^{-1}$) and phosphodiesters bonds (1,230–950 cm$^{-1}$) (Omoike & Chorover 2004) were also found. Due to the lack of detectable bands in the region between 1,000 and 700 cm$^{-1}$ characteristic for phosphate-sugar backbone vibrations (Socratess 2001), the peaks at 1,076 and 1,055 cm$^{-1}$ were assigned to polysaccharide vibrations rather than phosphodiesters bonds (Figure 4a, Figure S3).

**Raman spectroscopy**

The results confirmed the proteinaceous nature of the cement containing a significantly higher content of β-sheet structure than β-tum, α-helix and random coil (1,680–1,665 cm$^{-1}$) (Jiskoot & Crommelin 2005). The proteins contained a significant amount of phenylalanine (1,003 cm$^{-1}$ and 1,606 cm$^{-1}$) (Jiskoot & Crommelin 2005; Severcan & Haris 2012) and mostly buried tyrosine residues (855 and 832 cm$^{-1}$) (Severcan & Haris 2012). Bands for L-DOPA (735–730 cm$^{-1}$) (Ooka & Garrell 2000) and disulphide bridges (550–500 cm$^{-1}$) (Severcan & Haris 2012) were not found by Raman spectroscopy indicating an absence or an amount below the sensitivity of the method (Figure 4b). L-DOPA was also not detected on staining the cement with the Arnow method (Figure S2b).

**Discussion**

*D. fascicularis* is the only barnacle which produces gas-filled cement, thereby allowing the animal to float. The animals occur either individually or in clusters attached to flotsam or to the cement-buoys of conspecifics. The cement consisted of 8% dry matter and had a high water content of 92% in total, which fulfilled the definition of a hydrogel (Zavan et al. 2009). Other adhesives have also been classified as hydrogels, for example the gastropod adhesive gels (Smith 2006), the adhesive skin exudates of the Australian frog *Notaden bennetti* (Graham et al. 2005), the prey capture glue of the velvet worm *Euperipatoides* sp. (Graham et al. 2013) and the egg attachment glue of the moth *Opodiphthera* sp. (Li et al. 2008), but none of these have a foam-like structure as found in *Dosina*.

In agreement with the organic nature of the cement and the results of the adhesives of other barnacle species (Berglin & Gatenholm 2003; Sangeetha et al. 2010) the EDX spectra of the cement float of *D. fascicularis* showed high counts of carbon (C), oxygen (O) and nitrogen (N). However, in *D. fascicularis* the elements were distributed unevenly, unlike the cement of *Amphibalanus amphitrite* where high amounts of C, N and O were concentrated in rod-shaped structures (Sullan et al. 2009). Sulphur (S), magnesium (Mg) and small amounts of calcium (Ca), phosphorus (P) and potassium (K), presumably originating from seawater, were detected in the EDX spectra of the cement, but not in the line scans and dot mappings. This indicated that the quantities of these elements were near the detection limit of the EDX-system, which was 0.1 wt %. Interestingly, NaCl was not seen in the EDX spectra. The reason could be that the cement was rinsed in fresh water before it was used for EDX investigations and NaCl was washed away.

High amounts of Ca have been found in the cement of balanoid barnacles with a calcareous base (Walker 1972; Sangeetha et al. 2010). It is possible that Ca in the cement of these species came from the basal plate and/or the edge of the calcareous shell (Sangeetha & Kumar 2011). This kind of distortion could be ruled out in *D. fascicularis* because this goose barnacle has a membraneous attachment disc and its calcareous plates do not come into contact with the cement.

Although sulphur was identified in the EDX spectra of the cement of *D. fascicularis* no disulphide bridges...
Figure 4. FTIR and Raman spectra of dry and wet D. fascicularis cement. (a) FTIR spectrum of the cement. The region between 4,000 and 500 cm$^{-1}$ is magnified. Blue: sample equilibrated in D$_2$O; Red: dry sample. (b) Raman spectrum of the cement. The region between 4,000 and 480 cm$^{-1}$ is magnified. Blue: sample equilibrated in D$_2$O; Red: dry sample.
could be detected with Raman spectroscopy. In acorn barnacle cement, disulphide bonds are known to stabilise the protein complex and they contribute to its insolubility (Naldrett 1993; Kamino et al. 2000). Disulphide bonds are also commonly found in the adhesives of echinoderms, mussels, snails and sabellariid polychaetes (Benedict & Waite 1986; Flammang et al. 1998; Smith et al. 1999; DeMoor et al. 2003; Zhao et al. 2005). Sulphur can also occur as sulphated polysaccharides as seen in the adhesive of the sandcastle worm Phragmatopoma californica (Wang & Stewart 2013). A small distinct peak at 1,055 cm\(^{-1}\) in the FTIR spectrum of the D. fascicularis cement equilibrated in D\(_2\)O corresponds to polysaccharide vibration, which suggests that some glycosylated proteins occur in the cement. This was confirmed by the PAS method and the total carbohydrate analysis.

With the EDX system, phosphorus was detected in small amounts in the cement of D. fascicularis, which has only been reported for other barnacle cement by Walker (1972). Phosphorus is required as an activation constituent for post-translational modification to form phosphoserine, which plays an important role in the formation of a strong adhesive. This component was also found in the adhesives of caddis flies, mytilid mussels, sandcastle worms and the Cuvierian tubules of the sea cucumber as well as in kelp spore adhesive (Waite & Qin 2001; Zhao et al. 2005; Flammang et al. 2009; Stewart & Wang 2010; Petrone et al. 2011). However, phosphoserines were not found in the adhesive system of barnacles (Kamino 2010) and could not be chemically verified in D. fascicularis. Also L-DOPA, a post-translational modification of tyrosine, has never been identified in barnacle cement (Naldrett 1993; Kamino et al. 1996). This result was confirmed by the Arnow staining and the FTIR and the Raman spectra of the cement of D. fascicularis.

The adhesives of barnacles, mussels and sandcastle worms consist mostly of proteins (Walker & Youngson 1975; Kamino et al. 1996; Zhao et al. 2005; Silverman & Roberto 2007; Stewart et al. 2011), while the temporary adhesives of echinoderms and gastropods are made up of proteins and carbohydrates (Flammang 2006; Smith 2010). Distinct amide I, II and III bands in the FTIR spectrum and an amide I band in the Raman spectrum indicated a proteinaceous cement in D. fascicularis, as confirmed by the elemental analysis. The protein content of 84\% was higher than reported previously (75.9\%, Barnes & Blackstock 1974). This may be due to improved methods or to some individual variation. It is interesting that the protein content of the cement varies between acorn and goose barnacles and also in the different species of these two groups. Almost the same protein content as in D. fascicularis was found in Balamus hameri and B. crenatus (Walker 1972). The cement of the closely related goose barnacle Lepas anatifera (Walker & Youngson 1975) and that of the acorn barnacle Megabalanus rosa (Kamino et al. 1996) had a higher protein content (>90\%) than D. fascicularis.

According to Barlow et al. (2010), barnacle cement is largely composed of fibrillar proteinaceous material. Fibrous structures were identified by microscopic analyses in the cement of acorn barnacles (Wiegemann & Watermann 2003; Dickinson et al. 2009; Sullan et al. 2009) and the goose barnacle D. fascicularis (Zheden et al. 2012). One fibrillar proteinaceous structure associated with bioadhesion in barnacles is amyloid (Kamino 2008; Sullan et al. 2009; Barlow et al. 2010). In D. fascicularis, amyloid was detected by histochemical methods in the cement glands and in the cement (McEvilly 2011) but it was not found in the closely related barnacle Lepas anatifera (Jonker et al. 2012). The FTIR spectrum of D. fascicularis cement indicated the probability of a mixture of cross, parallel and antiparallel \(\beta\)-sheets due to major overlap of the relevant areas for amyloid-like and \(\beta\)-sheet structures (1,610–1,630 cm\(^{-1}\) vs 1,620–1,640 cm\(^{-1}\)), which could not be further specified by processing the amide I region via deconvolution (Barlow & Wahl 2012) (not shown). The \(\beta\)-sheet structures were previously found to be a feature of barnacle adhesives (Sullan et al. 2009; Burden et al. 2012). Furthermore, the FTIR spectra of wet and dry cement differed in the amide I band shift (Figure S3), which was most likely due to refolding of the secondary structure caused by dehydration (Hédoux et al. 2012; Hartwig et al. 2013).

The amino acid composition of the cement of D. fascicularis differed from earlier data by Barnes and Blackstock (1974, 1976), mainly in the smaller quantities of alanine, glycine and serine and the larger quantity of tyrosine. Tyrosine ring vibration is also indicated at 1,517 cm\(^{-1}\) in the FTIR spectra (Barth & Zscherp 2002) and as doublets at 833 and 855 cm\(^{-1}\) in the Raman spectra (Severcan & Haris 2012). Comparing the Dosima cement with that of L. anatifera, M. rosa, B. hameri and B. crenatus (Walker 1972; Walker & Youngson 1975; Kamino et al. 1996), the main differences are the smaller amounts of alanine, glycine, histidine, lysine and serine. The values of phenylalanine in the Dosima cement were higher, which was confirmed by Raman spectroscopy. Comparing the Dosima cement with that of B. hameri and B. crenatus (Walker 1972), the amount of valine was considerably higher and that of cystine and proline smaller (see Table 1). The high amounts of hydrophobic amino acids found in the cement of D. fascicularis agree with the findings in the cement of B. crenatus, B. perforatus and M. rosa. Naldrett & Kaplan (1997), Kamino (2010) and Kamino et al. (2012) stated that hydrophobic interactions rendered the cement matrix insoluble and made it resistant to decomposition by
marine bacteria (Naldrett 1993). Both qualities are particularly important for *Dosima* cement because the cement needs stability against mechanical impact while drifting in the sea (Zheden et al. unpublished). Because, unlike other barnacles, an excess amount of cement is produced by the animal, most of the cement surface is exposed to the environment and thus also to bacteria. Kamino (2008) reported that the major bulk proteins of barnacle cement are hydrophobic, whereas proteins for surface functions are hydrophilic. Barlow et al. (2009) indicated that the cement of live barnacles is mainly hydrated and therefore hydrophilic in its natural state. The isoelectric point of the observed cement proteins of *D. fascicularis* ranged from pH 3.5 to 6.0, indicating that mainly acidic proteins could be solubilised.

Indistinct protein bands with a molecular weight from <10.0 to 90.0 kDa were found in the cement of *D. fascicularis* by Barnes & Blackstock (1976). In the present study protein bands with a molecular weight between 47.4 and 250.0 kDa were identified. N-terminal sequence analysis by EDMAN degradation failed due to blocked termini of the proteins (data not shown). The reason for the N-terminal blocking can be by natural processes as protection for example against bacterial proteolytic enzymes or because of an undesirable effect of the sample preparation (eg by impurities of solubilisation or PAGE chemicals) (Wellner et al. 1990).

*De novo* sequencing was originally performed mainly manually with the help of database search, but is now commonly carried out by computational systems using algorithms to reduce time and costs (Ma & Johnson 2012). In the present study a set of 20 peptide sequences was identified (five of each analysed Dcfp). A NCBI blastp search of each of the peptides showed no results that allowed definite conclusions to be drawn about the analysed Dcfp or the underlying mechanisms of the adhesion process. In addition, it must be taken into account that only some of the solubilised proteins were analysed. Some of the identified peptides showed similarities to the barnacle cement proteins (cp) (Kamino 2013). At least 14 sequence hits were found for cp100 k, three hits for cp19 k and two for cp20 k and no hit for cp58 k or cp52 k. Markedly, the most abundant cement protein cp100 k (Kamino 2013; Lin et al. 2014) was also found in the present study with most matches (but not with the lowest E-values).

The NCBI blastp showed more than 20 matches for the protein MULTIFUNCin. This ~1,500 amino acid large glycoprotein (accession no: AFY13480) functions within the biomineralisation of barnacle shell and as attractant for the larvae of the barnacle. The latter applies also to *Dosima* whose larvae settle on the cement of conspecifics. However, MULTIFUNCin was not mentioned in the context of cement formation (Ferrier 2010).

The poor coverage of the database searches used could indicate unexpected post-translational modifications or peptide mutations (Ma & Johnson 2012), but it could also imply that in addition to the known cement proteins some unknown proteins are part of the cement. The applied MS/MS techniques can be used as the basis for understanding the *Dosima* cement as a natural underwater adhesive.

Understanding more about the *Dosima* cement could lead to the production of non-toxic artificial glues for medical and technical purposes (Smith & Callow 2006; von Byern & Grunwald 2010). In a follow-up study, it is planned to use peptide sequence tags to identify the full-length proteins by genome or transcriptome sequencing, or by RT-PCR methods (Hennebert et al. 2012; Lin et al. 2014). Furthermore, the peptides characterised in this study will be used to identify possible inorganic surface binding sequences in a combined computational and experimental approach as described by Steckbeek et al. (2014). The peptides can also be the basis for designing fluorescent-labelled nucleic acids for *in situ* hybridisation probes to localise RNA sequences (Wang & Stewart 2012). In addition, anti-peptide antibodies will be raised to localise the protein using immuno histochemistry approaches.

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References


IV. Manuscript 3

Mechanical properties of the cement of the stalked barnacle

*Dosima fascicularis* (Cirripedia, Crustacea)

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Personal contributions of Vanessa Zheden:

Collection of the material

Sample preparation for TEM and SEM

Cutting and staining of ultra-thin sections

TEM and SEM imaging and documentation

Performing micro-indentation experiments and tensile tests

Creation of graphs, box plots and performance of statistical analyses

Manuscript preparation
Mechanical properties of the cement of the stalked barnacle *Dosima fascicularis* (Cirripedia, Crustacea)

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**Abstract**

The stalked barnacle *Dosima fascicularis* secretes foam-like cement, whose amount usually exceeds that produced by other barnacles. When *Dosima* settles on small objects, this adhesive is additionally used as a float which gives buoyancy to the animal. The dual use of the cement by *D. fascicularis* requires mechanical properties different from those of other barnacle species. In the float, two regions with different morphological structure and mechanical properties can be distinguished. The outer compact zone with small gas bubbles is harder than the interior one, and forms a protective rind presumably against mechanical damage. The inner region with large, gas-filled bubbles is soft. This study demonstrates that *D. fascicularis* cement is soft and visco-elastic. We show that the values of the elastic modulus, hardness and tensile stress are considerably lower than in the rigid cement of other barnacles.

**Key words:** adhesive, elastic modulus, hardness, tensile stress
Introduction

Sessile marine organisms secrete adhesives which are cured underwater and which remain durable in the water [1, 2]. The best-studied animals, producing a strong adhesive, are the invertebrates, such as barnacles, mussels and tubeworms [3].

Barnacles are among the most troublesome and dominant fouling organisms [4]. They settle as cypris larvae on any hard substratum, whether it is man-made like ships and bridges or organisms like crabs and turtles. After metamorphosis from the cyprid to the juvenile both acorn and stalked barnacles usually produce only a thin layer of permanent adhesive, the so-called cement, by which they adhere to the substratum. The stalked barnacle *Dosima fascicularis* (Ellis and Solander, 1786) is exceptional, secreting a large amount of foam-like proteinaceous cement which is produced by the cement glands and led through the stalk in a complex canal system. At the base of the stalk the cement is extruded through pores in the cuticle. The bubbles enclosed in the cement contain gas [5-7] whose nature is not known so far. It is assumed that it may be CO₂, a byproduct of metabolism, which is transported by the hemolymph and diffuses through the lining cells of the cement canals into the lumen of the ducts. The excretion of CO₂ together with the cement is doubly advantageous for the animal: it causes the formation of gas bubbles in the cement and contributes to the pH regulation in the hemolymph [8].

*D. fascicularis* mainly attaches to floating objects such as feathers, driftwood, seaweed and tar pellets [5, 7, 9, 10]. As the animal grows, the amount of cement increases and can subsequently enclose any small substratum to which it adheres. With this so formed float *D. fascicularis* drifts passively in the neuston [11]. Other animals, like marine snails of the family Janithidae, are also rafting in the water but their float is of different origin and consists of mucus bubbles [12, 13]. It is assumed that the float is derived from an egg mass which is modified for buoyancy [12]. The precise composition of this material has not yet been analysed. Apart from the use for locomotion foam-like materials serve for attachment and
protection. Marine mussels like *Mytilus californianus* and *M. edulis* adhere to the substratum by means of the proteinaceous byssus. The adhesive plaque of the byssus resembles a solid foam with a spongy inner structure and a fibrous surface matrix [14-16]. According to Grayson [17] a solid foam is defined as material “the apparent density of which is decreased substantially by the presence of numerous cells disposed throughout its mass”. The marine sandcastle worm *Phragmatopoma californica* builds protective tubes of solid foam [18] by gluing sand grains and parts of shells together with a proteinaceous cement [19, 20].

In addition, biofoams are frequently used by animals for the protection of brood. Examples are the nymphs of spittlebugs which secrete a froth, consisting of a proteoglycan and glycoprotein complex. The froth surrounds their body and thus protects them from e.g. desiccation [21]. Fish like the armoured catfish protect their eggs in floating mucus foam nests [22, 23]. Tropical frogs produce proteinaceous foams to protect their eggs and embryos against environmental challenges [24].

Little to nothing is known about the mechanical properties of the biofoams [e.g. 25, 26] and so far nothing is known about the mechanical qualities of the cement of stalked barnacles. After the detailed documentation of the morphology of the cement apparatus and the cement [8] and the study of the biochemical composition of the cement of *D. fascicularis* [27], it is the aim of this study to investigate the mechanical properties of this adhesive. Studies about the acorn barnacle cement show that its structure differs between different species and also within the same species depending on the substratum to which the animal is attached [2, 28]. The adhesive may be fibrous, globular or sponge-like [1, 2, 29, 30]. Accordingly mechanical properties of the cement differ. Among the best investigated qualities are adhesive tenacity, elastic modulus, and hardness. On hard substratum the adhesive tenacity or removal stress of the temporary adhesive of the cyprid (which is searching for a suitable place for attachment) and of the permanent cement of the juvenile is almost the same (around 0.2 MPa). Considerably higher tenacity values (around 0.9 MPa) were found in the permanent adhesive
of a settled cyprid and the cement of the adult [31, 32]. By using elastomeric coatings, the removal stress can be lowered and barnacles can easily be detached [30, 33, 34]. The substratum also influences the elastic modulus and hardness of the cement. These values are higher on non-metallic than on metallic substrata. In previous experiments, it was shown that acorn barnacles secrete more cement on low energy, polymeric surfaces to adhere firmly than on high energy surfaces like metal [2]. Compared to the cement of acorn barnacles the Dosima cement has a different structure [8] and according to the definition by Grayson [17] it is a solid foam. Its main function is to give buoyancy to the animal [9], apart from providing a reliable bond to the substratum. Therefore, it is to be expected that its mechanical properties differ from those of other barnacles whose only function is to adhere strongly to the substratum. In contrast to the thin and firm cement of acorn barnacles, the cement float of D. fascicularis appears soft and elastic.

In the present study, both the elastic modulus and hardness of the cement were measured using micro-indentation. Furthermore, the cement float was pulled apart in a tensile test until it ruptured, and the tensile stress was compared with the stress measured in acorn barnacles at the pull off from the substratum. The mechanical properties of the cement of D. fascicularis are interesting in comparison with those of acorn barnacles and in the context of the possible use of this adhesive in medicine and technology.

**Material and methods**

*Dosima fascicularis* which had been washed ashore was collected on the North-West coast of Denmark. The cement was removed from the animals and stored in sea water with a 2% antibiotic antimycotic solution (Sigma-Aldrich, Vienna, Austria) prior to the analyses.
Electron microscopy
The cement was fixed in 2.5% glutaraldehyde in 0.1 mol l\(^{-1}\) sodium cacodylate buffer with 10% (w/v) sucrose at pH 7.3 for 2 h. For scanning electron microscopy, cross sections of the pre-fixed cement floats were rinsed in distilled water, air-dried and coated with gold by an Agar B7340 sputter coater (Agar Scientific Ltd., Stansted, Essex, U.K.). The samples were examined in a Philips XL 30 scanning electron microscope (FEI and Philips, Eindhoven, NL) at 15 kV.

For transmission electron microscopy small pieces of pre-fixed cement were post-fixed in 1% osmium tetroxide in 0.1 mol l\(^{-1}\) sodium cacodylate buffer for 2 h. The samples were dehydrated in a graded ethanol series, and acetonitrile was used as intermediate medium before embedding in Agar Low Viscosity Resin. 60 nm sections were cut on a Reichert Ultracut-S microtome (Leica Microsystems, Vienna, Austria), stained with 0.5% uranyl acetate and 2% lead citrate. The sections were viewed in a Zeiss EM 902 transmission electron microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at 80 kV.

Micro-indentation experiments
The elastic (Young’s) modulus and the hardness of the *D. fascicularis* cement were measured by micro-indentation. The cement floats were cut into halves and the almost smooth surface and the inner foam-like region were measured in sea- and distilled water at room temperature. Also the surface of dried cement was measured. On each of these samples three indentations were performed on different spots. The samples were fixed in a metal frame, so that they could not drift away. In our experiments, the Basalt 01 microtribometer (Tetra GmbH, Germany) [35-37] was used. Indentations were performed using a glass sphere (3 mm diameter) fixed to a metal spring (figure 1a). The spring constant was calculated experimentally from the slope of the force-distance curve obtained on hard substratum (aluminum block). The effective Young’s modulus \(E\) and the hardness \(H\) of the cement
samples were defined from the fit of unloading part of the force-indentation depth curves according to the Hertz equation [38] (figures 3a, b):

$$ F = \frac{4}{3} \frac{E \sqrt{R}}{1 - \nu^2} \delta^{1.5} \quad \text{and} \quad H = \frac{F_{\text{max}}}{\pi R \delta_{\text{max}}}, $$

$R$ is the radius of the glass sphere, $\nu$ is the Poisson ratio, $\delta$ is the indentation depth caused by the applied force $F$, $F_{\text{max}}$ and $\delta_{\text{max}}$ are the maximal values of the applied force and indentation depth respectively. The software MATLAB 7.12.0 (The MathWorks, Inc., Natick, MA, USA) was used for the fit. Graphs, box plots and statistical tests were created in Sigma Plot 11.0 (Systat Software Inc, Bangalore, India and San Jose, CA, USA). Statistical analysis was done with the nonparametric Kruskal-Wallis one way analysis of variance on Ranks with Dunn’s multiple comparison procedure (significant set at $P < 0.05$). Data were fitted by linear regression and tested for normal distribution using a Shapiro-Wilk’s test.

**Tensile test**

In this experiment whole cement floats as well as parts of the outer region of the cement float were used. These parts were cut into cubes of about 10 x 10 x 0.2 mm. The samples were then fixed by two clamps (figure 1b), one clamp was fixed on a force transducer FORT 100 (World Precision Instruments, Sarasota, FL, USA) which pulled the sample with a constant speed of 200 $\mu$m/s. The cement was pulled in 5 mm steps, each followed by a resting period of around 8 s until the sample ruptured. The pulling force and distance were measured. Additionally, the strain at rupture was defined. Knowing the area (width multiplied by height) and the length of the cement samples, a stress–strain curve could be generated. The value of the stress $\sigma$ was obtained from the applied force $F$ divided by the cement cross-section area $A$ ($\sigma = \frac{F}{A}$) and the strain $\varepsilon$ from the extension $\delta$ divided by the length $l$ of the cement sample ($\varepsilon = \frac{\delta}{l}$). The graphs were created in Sigma Plot 11.0. The data for the relaxation (figure 8b) were fitted by a single exponential function and tested for normal distribution utilising Shapiro-Wilk’s test.
Results

Cement morphology

Dosima fascicularis occurred individually or in groups (figure 2a), producing a large amount of cement. The foam-like cement enclosed gas-filled bubbles of different size (figure 2b), which gave buoyancy to the animal. The cement was secreted in concentric layers around the stalk. The outer layers, forming a kind of rind, were narrow and contained small bubbles (figure 2c). Inside the float were mainly large bubbles. In the scanning electron microscope, the cement had a rough structure (figure 2d), in the transmission electron microscope it appeared fibrous with condensed zones, where the fibres aggregated (figures 2e, f). Some of these zones formed the frames of the bubbles.

Micro-indentation test

Typical curves of loading force versus indentation depth of the wet and the dry cement were shown in figures 3a and 3b. The force – indentation depth curves of two consecutive indentations at the same place of the float provided information about visco-elastic-plastic deformation of the cement during the loading/unloading process (figure 4). The plastic deformation is seen as a shift of the second indentation curve (gray) relative to the first indentation curve (black). The large difference in loading and unloading parts of the indentation curves demonstrates the pronounced viscose properties of the float. However, the main mechanical response of the float on the indentation is elastic.

The elastic modulus of the cement surface measured in sea water was 16.4 kPa ± 8.8, whereas in distilled water it was 11.6 kPa ± 5.3. In the inner region the modulus was 9.3 kPa ± 5.3 measured in sea water and 8.5 kPa ± 3.6 in distilled water. Statistically there was a significant difference between the different regions of the cement in the two media (P < 0.001, Kruskal-Wallis test). However, the pairwise comparison revealed that the inner regions were not
significantly different. The dry cement had a much higher Young’s modulus (0.76 MPa ± 0.87) than the cement under wet conditions (figure 5a).

The hardness of the cement surface measured in sea water was 2.5 kPa ± 1.2, whereas in distilled water it was 1.7 kPa ± 0.7. In sea water the inner region had a hardness of 1.5 kPa ± 0.9, in distilled water 1.3 kPa ± 0.4. As for the elastic modulus, the hardness differed significantly ($P < 0.001$, Kruskal-Wallis test), but the pairwise comparison showed that there was no significant difference between the inner regions in sea- and distilled water. The dry cement had the highest hardness values (39.0 kPa ± 23.2) (figure 5b).

The elastic modulus and the hardness were higher at the surface than in the inner zone of the wet cement. With an increasing indentation depth, the elastic modulus ($R^2 = 0.22$, Shapiro-Wilk’s test) and the hardness ($R^2 = 0.06$, Shapiro-Wilk’s test) decreased (figures 6a, b). In the inner region of the cement float no correlation between the indentation depth and the elastic modulus ($R^2 = 0.0002$, Shapiro-Wilk’s test) or hardness ($R^2 = 0.006$, Shapiro-Wilk’s test) was observed (figures 6c, d). In the dry cement the values of the elastic modulus ($R^2 = 0.47$, Shapiro-Wilk’s test) and hardness ($R^2 = 0.43$, Shapiro-Wilk’s test) decreased significantly with an increasing indentation depth (figures 6e, f).

### Tensile test

During pulling, the cement was elastically extensible to the point of rupture (figure 7). The tensile stress of the cement was lying below 0.2 MPa (figure 8a). During the resting period, the force decreased, indicating slow relaxation of the material (figures 8a, b). This showed the visco-elastic properties of the cement.

### Discussion

Many organisms use adhesives for a variety of purposes e.g. for attachment, defence or protection [18, 39, 40]. In marine animals the attachment can be permanent as in mussels,
transitory as in turbellarians or temporary as in echinoderms [41]. Barnacles use both temporary and permanent adhesion during their life cycle. The last larval stage, the cyprid, uses temporary adhesion to explore the substratum before settlement and the adult is permanently attached [32]. According to Yule and Walker [31] the cyprid used a low bond strength cement in comparison to the higher bond strength cement of the adult.

Normally barnacles deposit a thin layer (a few µm thick) of firm adhesive on high energy surfaces. Only when they adhere firmly on polymeric substrata with low energy they produce a thicker cement layer with sponge-like structure. Interestingly this spongy cement was harder and had a higher elastic modulus than the firm cement [2]. In contrast to all other barnacles Dosima fascicularis produces a large amount of foam-like cement which contains gas-filled bubbles. The cement is secreted in concentric layers around the stalk and the attached substratum [8]. It is known that the layered structure of barnacle cement is the result of cyclic secretion during the growth of the animal [30, 42, 43]. Sun et al. [30] described the multilayered structure of the adhesive plaque of Balanus eburneus and B. variagatus. In these species the elastic modulus, in the range of 0.01-100 MPa, increased from the outer to the inner layer. In contrast to the Balanus cement, the surface of the Dosima cement had a higher elastic modulus and was harder than the inner region (figure 6). A reason for this may be that the salt in the sea water hardens the surface of the cement. In experiments we could show that the elastic modulus and hardness of the cement surface were higher in sea water than in distilled water. In addition, the outer narrow layers formed a rind, containing only small bubbles (about 11 µm in diameter), in comparison to the inner region where the elongate bubbles (up to 2460 µm in length) predominated [8]. This structure will give greater mechanical stability and stronger protection to the surface region. The rind, which acts as interface towards the environment, is prone to any mechanical- or UV-damage and possible dehydration. Similar structural and mechanical characteristics were also observed in the cement of Phragmatopoma californica, where the smallest bubbles were at the interface to the
surrounding water [18]. These authors suggested that the highest elastic modulus would be at the interface. Also the inner spongy plaque matrix of the mussel *Mytilus edulis* became increasingly dense toward the outside [14]. This seems to be a common phenomenon in the adhesives of aquatic animals.

The cement of barnacles is generally fibrous [8, 29, 44-46] and visco-elastic [2, 30]. Visco-elasticity is known to be a property of many, if not all, biological materials [47]. It was described of natural fibrous composites, like the cuticle of the attachment pads of Orthoptera [35, 37]. Also non fibrous materials like those of echinoderm tube foot discs [48] and the adhesive gels of gastropods [49] had visco-elastic properties. Visco-elasticity of the cement of *D. fascicularis* may be necessary to protect the gas-filled bubbles inside the float from rupture by any fast and strong mechanical impact e.g. caused by water current and waves.

Most values of the elastic modulus of the wet cement of *D. fascicularis* were in the range of 5-20 kPa. Investigations of the cement of the acorn barnacle *Amphibalanus* spp. showed that the elastic modulus of the cement was higher than that of *D. fascicularis*. The modulus of elasticity of the cement of *A. amphitrite* measured by Ramsay *et al.* [50] with the punch test apparatus was between 2.9 GPa and 6.5 GPa. Sullan *et al.* [1] performed AFM-nanoindentations on different structures of the cement of the same species and got lower values of the elastic modulus ranging from 0.2 to 90 MPa. The difference of these results may be due to the different methods used. Sangeetha and Kumar [2] analysed the cement of *A. reticulatus*, growing on metallic and non-metallic substrata, using a nanomechanical testing system. The hardness and the elastic modulus of the cement were higher on non-metallic (*H* = 52.6 MPa and *E* = 1.2 GPa) than on metallic substrata (*H* = 8.7 MPa and *E* = 0.4 GPa) and again lower than those of the *Dosima* cement. These authors reported that barnacles needed more cement to adhere firmly to non-metallic substrata than to metal and that detachment of barnacles from metallic surfaces was generally more difficult than from non-metallic ones.
The acorn barnacles described above, had a calcareous base. It cannot be ruled out that parts of the hard calcareous base were included in the measurements. In our experiments, only the pure cement, free of any animal tissue and free of any substratum, was investigated. In the indentation experiments, we selected indentation depths at least 10-fold lower than the sample thickness, in order to prevent contribution of the stiff support to the results of our measurements.

Our results revealed that the *D. fascicularis* cement is a soft biological material. Its elastic modulus was in the same range as that of other animal adhesive structures such as the tube foot discs of echinoderms (3-140 kPa) [48], the adhesive pads of ensiferan insects (25-100 kPa) [37] and the adhesive secreted by the Australian frog *Notaden bennetti* (170-1035 kPa) [51]. Very hard marine biomaterials were the adhesive secreted by the serpulid *Hydroides dianthus* (about 3 GPa [52]) and the byssal threads of *Mytilus* mussels. The protective outer cuticle of the byssal threads of *Mytilus californianus* and *M. galloprovincialis* had a modulus of about 1.7 GPa and a hardness of around 0.1 GPa. The elastic modulus and the hardness of the cuticle were 4-6 times greater than that of the inner collagen core of the byssal threads [25].

The dry cement of *D. fascicularis* had an elastic modulus of approximately 0.8 MPa, and was thus much harder than the wet cement. It is known that dehydration hardened the originally soft barnacle adhesive [30]. Desiccation also hardened biological materials, like insect cuticle or bones [53, 54].

The adhesive strength or removal stress of acorn barnacles was, like the elastic modulus and the hardness, influenced by the substratum. Unlike the elastic modulus and hardness, the tenacity (adhesive strength) was higher on "natural", hard surfaces (around 0.9 MPa) than on polymeric substrata (< 0.1 MPa) [32, 33, 55]. *D. fascicularis* mostly settles on small, organic floating objects, which may be gradually enclosed in the cement float. Therefore, the cement could not be removed from the substratum and the removal stress could not be measured.
order to compare the acorn barnacle cement with that of \textit{D. fascicularis}, the \textit{Dosima} cement was pulled apart until rupture. The tensile stress determined in such an experiment was below 0.2 MPa. Accordingly, the tensile stress of the \textit{Dosima} cement was lower than the removal stress of the cement of acorn barnacles attached to natural stiff substratum, but it was slightly higher than that of barnacles attached to polymeric substrata.

\textbf{Conclusions}

The cement of \textit{Dosima fascicularis} is a soft biological material and like that of other barnacles fibrous and visco-elastic. The values of elastic modulus, hardness and tensile stress were much lower than in the rigid cement of acorn barnacles investigated so far. A physical explanation for these differences is the foam-like structure of the \textit{Dosima} cement caused by the gas-filled bubbles. An ecological explanation could be the differing living conditions of acorn barnacles and \textit{Dosima} with the partly different use of the adhesive. In contrast to the gregariously settling acorn barnacles, which are firmly attached to the substratum, \textit{D. fascicularis} is either singly or in small numbers attached to floating objects or drifting through the sea autonomously with a cement float. For this lifestyle the \textit{Dosima} cement has to be resilient to withstand mechanical impact in the water, not hard as in other barnacles and – very important – the float must not be permeable for water and gas. The great elasticity enhanced by the foam-like structure gives the cement damping properties. In addition, this special structure of the cement is more economical for the animal than a solid structure. It saves material and thus energy.

The shock-absorbing properties combined with the expected biocompatibility of the \textit{Dosima} cement make it interesting for possible applications in orthopaedics. Its structure and the assumed biodegradability make it like other biofoams \cite{56, 57} perfectly suitable as three-dimensional scaffolds for tissue growth and wound healing. Besides the possible application in medicine the \textit{Dosima} cement could also be used in technology. Its foam-like weight saving
structure in conjunction with the fact that the cement cures and is durable underwater could make it an appropriate material for construction works in wet environment.

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References


**Figure captions**

**Figure 1.** (a) Schematic drawing of the indentation experiment setup. The glass sphere was attached to a spring, which was driven by a motor and indented the cement. (b) For the tensile experiment, a part of the outer region of a cement float (c) was fixed between two clamps. The upper clamp attached to the force transducer (ft) was pulling the sample with a constant speed while the lower clamp was fixed.
Figure 2. (a) Stranded aggregation of *Dosima fascicularis* connected by a single cement float (c). (b-d) Scanning electron micrographs. (b) Cross section of a cement float. The outer zone forming a rind (r) contained mainly small bubbles, whereas in the inner region of the float large bubbles (b) were dominant. (c) Higher magnification of the rind with narrow layers (arrows). (d) On the layers and the inner lining of the bubbles the cement showed some roughness. (e-f) Transmission electron micrographs. (e) The cement had a fibrous structure.
In the region, where the fibres were condensed, irregular lines were seen. Scale bars: (a) 1 cm; (b) 500 µm; (c) 100 µm; (d) 5 µm; (e) 2 µm, (f) 5 µm.

**Figure 3.** Typical force *versus* indentation depth curves of the cement of *Dosima fascicularis*. 
(a) The cement surface measured in sea water. (b) The surface of the dry cement. The solid line indicates the fit of the indentation data with the Hertz theory. (*E* = elastic modulus)

**Figure 4.** Two consecutive indentations, applied with the same force, measured on the same place at the surface of a wet cement float of *Dosima fascicularis*. The second indentation (grey) demonstrated a visco-elastic-plastic deformation when compared to the first indentation (black).
Figure 5. The elastic modulus (a) and the hardness (b) of the surface and inner region of the cement float of *Dosima fascicularis* measured in sea water and distilled water as well as of the surface of the dry cement. Box plots show the median value (line), the ends of the boxes define the 25th and 75th percentiles and the error bars the 10th and 90th percentiles. The outlines are illustrated as black dots. The difference between the wet and dry cement was obvious, therefore only the differences between the different regions under wet conditions were analysed using Kruskal-Wallis one way analysis of variance on Ranks. (a, b) \( P < 0.001 \).
Figure 6. Correlation between the indentation depth and the elastic modulus \((a, c, e)\). Correlation between the indentation depth and the hardness \((b, d, f)\). \((a, b)\) Surface of the wet cement (in sea water, \(n = 116\)). \((c, d)\) Inner region of the wet cement (in sea water, \(n = 46\)). \((e, f)\) Surface of the dry cement (\(n = 40\)). The data were fitted by linear regression and tested for normal distribution using a Shapiro-Wilk’s test. \((a)\) \(y = 37.85 - 0.11x, R^2 = 0.22, P < 0.0001;\) \((b)\) \(y = 4.002 - 0.007x, R^2 = 0.06, P < 0.0001;\) \((c)\) \(y = 9.77 - 0.002x, R^2 = 0.0002, P < 0.0001;\)
(d) $y = 1.063 + 0.002x$, $R^2 = 0.006$, $P < 0.0001$; (e) $y = 2.69 - 0.02x$, $R^2 = 0.47$, $P = 0.07$; (f) $y = 94.18 - 46x$, $R^2 = 0.43$, $P = 0.1$ ($R^2$ = coefficient of determination).

**Figure 7.** The curve shows a force – distance dependence during pulling the cement (in this case, a parallelepiped-shaped sample of the outer region of the float was used) until it ruptured.

**Figure 8.** (a) The stress-strain curve obtained in the pulling experiment shows relaxation (see b) of the cement, when the pulling was stopped for around 8 s. (b) Relaxation (decrease in the interacting force) of the cement, lasting for several seconds, was observed during the resting period. The relaxation process was strongly pronounced during deformation of the cement. It was represented by a single exponential function and tested for normal distribution utilising Shapiro-Wilk’s test. $f = 0.6 - 0.2e^{-0.4x}$, $R^2 = 0.88$, $P < 0.0001$ ($R^2$ = coefficient of determination).
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Characterization of cement float buoyancy in the stalked barnacle

*Dosima fascicularis* (Crustacea, Cirripedia)

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Sample preparation for SEM and LM
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Performance of all experiments
Creation of graphs, histograms and performance of statistical analyses
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Characterization of cement float buoyancy in the stalked barnacle

*Dosima fascicularis* (Crustacea, Cirripedia)

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**Abstract**

*Dosima fascicularis* is the only barnacle which can drift autonomously at the water surface. The animal secretes a large amount of foam-like cement which often overgrows the substratum to which it is attached. When several individuals share one float, their size and not their number are crucial for the production of both volume and mass of the float. The gas content within the cells (bubbles) of the foam gives buoyancy to the whole float. The higher the gas volume, the greater is the positive buoyancy. The cement consists of more than 90% water and the gas volume is on average 18.5%. Our experiments demonstrate that the intact foam-like cement float is sealed from the surrounding water. The effective density of the cement floats without gas is greater than that of the sea water.

**Key words:** adhesive, buoyancy, foam, biological material, Arthropoda
Introduction

Barnacles (Cirripedia Thoracica) are sessile, marine Crustacea with free-swimming larval stages. The adult barnacle attaches permanently with its proteinaceous adhesive, the so-called cement, to stationary hard materials like rocks or to natural and man-made floating objects. Lepadomorph stalked barnacles are typical rafting organisms [1]. Their pelagic larvae colonise any floating objects like driftwood, macroalgae, plastic or tar pellets [2-4]. They even settle on living animals, e.g. sea snakes [5, 6], turtles [7], birds [8], and seals [9]. With this strategy, barnacles can extend their habitat. The normally sedentary animals turn into “hitchhikers” which raft through the sea [9, 10]. Generally lepadomorph barnacles secrete a thin layer of cement for attachment. One of them, *Dosima (Lepas) fascicularis* (Ellis and Solander, 1786), has evolved special adaptations. This animal produces a large amount of foam-like cement with enclosed gas-filled bubbles [11]. The gas is presumably CO₂, a metabolic product [12]. The cypris larva of *D. fascicularis* settles mainly on small floating objects, like bird feathers or algae. After metamorphosis of the cyprid and as the animal grows, the amount of cement increases. It may overgrow the substratum and thus form a float [13, 14] which gives buoyancy to the animal and enables it to drift autonomously just at the water surface [1]. The cement is produced by cement glands in the upper part of the stalk, transported together with the gas through the cement canal system and secreted through pores on the stalk base [12].

The phenomenon of positive buoyancy is known of other marine organisms too like some snails, cnidarians and brown algae. They also produce their own floating devices with which they drift through the water. The snails of the family Janthinidae secrete a float of mucus bubbles [15, 16]. With that float they drift and hunt in the neuston in contrast to the related benthic species. Neustonic cnidarians like *Velella velella* and *Physalia physalis* have one gas-filled bladder which acts as a sail allowing them to float on the water surface [17, 18]. Brown algae like *Fucus vesiculosus* have gas-filled floats, called pneumatocysts, which give positive
buoyancy to the blades [19, 20]. By that they are exposed to more sunlight which improves their photosynthetic activity.

Most studies on such biological floats deal with the ecology of drifting or rafting organisms [1, 10, 21], but to the best of our knowledge nothing is known about positive buoyant properties of these structures. After having dealt with some mechanical properties [22], the aim of this study was to investigate the unusual function of the cement of *Dosima fascicularis* with special emphasis on the positive buoyancy of the cement float, the gas- and the water content as well as the volume-weight correlation.

**Material and methods**

Individuals of the stalked barnacle *Dosima fascicularis*, which had been washed ashore, were collected on the north-west coast of Denmark. The animals occurred individually (figure 1a), in groups on the same cement float (figure 1b) or attached to small floating objects (e.g. feathers). For our investigations we used colonies of two to seven animals attached to one float. All animals of one colony had a capitulum length between 2 and 3 cm. For the experiments only the cement floats were used. Prior to the analyses the cement was separated from the animals and stored in sea water with a 2% antibiotic antymycotic solution (Sigma-Aldrich, Vienna Austria).

**Microscopy**

Razor blade sections through whole cement floats were photographed with a Lumix DMC-GH1 camera (Panasonic, Hamburg, D) mounted on a MZ3000 stereo microscope (Micros Austria, St. Veit/Glan, A). For scanning electron microscopy, cross sections of the float were fixed in 2.5% glutaraldehyde in 0.1 mol l⁻¹ sodium cacodylate buffer with 10% (w/v) sucrose at pH 7.3 for 2 h and rinsed in distilled water. After air-drying the cement samples were coated with gold by an Agar B7340 sputter coater (Agar Scientific Ltd., Stansted, Essex,
U.K.) and examined in a Philips XL 30 scanning electron microscope (FEI and Philips, Eindhoven, NL) at 15 kV.

**Mass, volume and water content**

Ten cement floats were weighed under wet conditions and their volumes were determined by water displacement. For measuring the water content of the cement the floats were wiped with tissues to remove any excess water. Two floats were then air dried for two days, in addition three floats were dried in the oven at 65°C overnight. Afterwards the dried cement floats were weighed again. The water content was calculated by subtracting the dry mass from the wet mass. Some salts of sea water may still have been on the surface of the dried floats.

**Buoyant force and gas volume**

Ten cement floats were placed one after the other in a 100 ml beaker filled with sea water. Each float was totally submerged to a depth of 1 cm below the water surface by a thin wire-frame attached to a force transducer using a motorised micromanipulator (FORT-100 and DC3001R, World Precision Instruments, Sarasota, FL, USA) (figure 1c). The floats were kept underwater for about 20 s and their buoyant force was measured. The data were collected with the software AcqKnowledge 3.7.0 (Biopac Systems, Inc., Goleta, CA, USA). Afterwards, volume, mass and buoyant force of the cement floats were determined. The volume of the gas $V_g$ inside the cement float was calculated using the following equation

$$V_g = \frac{F + M(1 - \rho_w/\rho_c)}{\rho_w - \rho_g}.$$  

$F$ was the positive buoyant force, $g$ the gravitational acceleration equals 9.81 m/s$^2$ and $M$ was the mass of the dried cement float. The density of the sea water $\rho_w$ (1018.65 kg/m$^3$) was calculated as mass divided by volume. Because of the irregular internal structure of the cement float (due to the greatly differing size of the gas bubbles) and the resulting uncertainty...
in determining the density of the cement we used for our calculations the standard value \( \rho_c = 1350 \text{ kg/m}^3 \) for the density of proteins [23, 24]. For the density of CO\(_2\) within the bubbles [12] we used the standard value \( \rho_g = 1.977 \text{ kg/m}^3 \).

**Reduced pressure experiments**

In a vacuum-desiccator cement floats were placed in a beaker filled with sea water. Two of them were exposed to a pressure of 700 mbar and two others to 30 mbar. 30 mbar were reached after about 20 min. During pressure reduction the floats were observed to detect any structural changes and any escaping gas bubbles. After achievement of the desired pressure the floats were kept at this pressure for five minutes. The buoyant force of the floats at atmospheric pressure (996 mbar) was measured before and after the reduced pressure treatment.

**Statistical analyses**

The regression calculations were done via a random bootstrap approach [25, 26] (with 10,000 iterations) by the program routine MUREG from the software package of ‘computer intensive statistics’ [27]. Graphs and histograms for all experiments were made in Sigma Plot 11.0 (Systat Software Inc, Bangalore, India and San Jose, CA, USA).

**Results**

**Cement morphology**

The cement was secreted in concentric layers around the stalk and the attachment site (figure 2a). Large gas-filled bubbles were mainly found in the inner region of the cement float. Small, round bubbles arose throughout the float, but dominated near the surface of the float forming a kind of rind (figure 2b).
Mass, volume and water content

There was a significant correlation between the volume of the cement float of *Dosima fascicularis* and its wet mass ($R^2 = 0.96$, $P < 0.001$). The greater the volume, the heavier was the float (figure 3). The number of animals attached to the same cement float did not influence mass and volume of the float. Only the size of the animals was crucial. For example, the float with four animals attached was heavier and bigger than the floats with seven animals (table 1).

The water content of the cement floats was over 90% (w/w), no matter whether they had been air dried or oven dried (figure 4a). When the dried cement was immersed in sea water again, it was still floating. Within a few minutes the cement float got heavier because of water uptake. After three days in water the air dried cement floats had more than the threefold dry mass, but they did not return to the original wet mass. Interestingly, the oven dried floats did not take up as much water as the air dried ones (figure 4b).

Buoyant force and gas volume

The volume of the gas was linearly dependent on the volume of the float ($R^2 = 0.65$, $P = 0.018$) (figure 5a). In essence, the bigger the float, the higher was the gas volume. The gas volume fraction in a float was on average $18.5\% \pm 6.7$ ($n = 10$). A typical graph of the measured positive buoyant force is shown in figure 5b. In this figure the force required to hold the cement float underwater was 9.05 mN. The volume of the gas inside the float and the buoyant force were significantly correlated ($R^2 = 0.99$, $P < 0.001$) (figure 5c). Clearly, the higher the gas content in the float, the more positive buoyancy it had.

Reduced pressure experiments

For the pressure experiments two cement floats, kept in sea water, were exposed to 700 mbar, two others to 30 mbar ambient pressure. There was almost no difference between the buoyant
force, measured before and after treatment with 700 mbar underpressure (figure 6a: floats 1 and 2). During pressure reduction gas bubbles were observed leaking out and accumulating at the surface of the float around 400 mbar (figure 6b arrows). At that pressure the cement float started to crack. Below 250 mbar most bubbles in the cement had burst and water filled the voids. Immediately after ambient pressure reduction to 30 mbar, the cement floats had still some positive buoyancy, but in comparison with the floats exposed to 700 mbar underpressure, the buoyant force was much lower (figure 6a: floats 3 and 4). As a consequence, the floats, which were exposed to 30 mbar, sank a few hours after the experiment. Due to the water uptake the mass of the floats increased. After five days the cement was >25% heavier than measured immediately after the underpressure experiment.

Discussion

The stalked barnacle Dosima fascicularis is unique among the barnacles being able to secrete its own float. It can even form colonies attached to a central float [28]. Interestingly, volume and mass of the float are primarily correlated with the size of the attached animals and not with their number. The colonies investigated in this study contained barnacles of similar size. This is presumably the result of several cypris larvae having settled on the same substratum during the same reproductive period. The increasing cement of the adult eventually formed one joint float. This was also previously suggested by Ryan and Branch [28].

Water content

The original wet cement of D. fascicularis had a water content of more than 90% [see also 29]. Interestingly, after air drying the cement took up more water than when it was oven dried. A possible explanation is that the protein structure of the cement changes due to irreversible protein denaturation during heating up to 65°C. Another reason for the difference in water uptake could be that the process of air drying is slower and thus gentler than oven drying.
Therefore the air dried material did not shrink that much and water could get more easily into it again. The reason that the dried floats did not get their original mass after rehydration might be that the immersion time was too short.

**Buoyancy**

Floating is a typical feature of lepadomorph barnacles. *Lepas anatifera* or *L. testudinata* attach with a thin layer of cement to large and highly buoyant objects like kelp or plastic [30]. In contrast *D. fascicularis* normally settles on small floating objects, which are often overgrown by the cement. By developing its own foam-like cement full of gas bubbles, the *D. fascicularis* cement maintains positive buoyancy in accordance with the growth of the animal throughout its life time [2, 28].

For the estimation of the cement density we used the value 1350 kg/m$^3$ of protein density [23, 24]. According to this value the density of the proteinaceous cement was higher than that of sea water (1018.65 kg/m$^3$). Consequently the cement without gas bubbles would sink. In principle, the greater the volume of the float the bigger was the gas volume, but the gas content of the floats varied between the *D. fascicularis* colonies (see table 1). As to be expected the floats with less gas content had lower positive buoyancy than floats with higher gas content. The gas volume of the float and thus its positive buoyancy could also depend on the object to which the float is attached. The use of floating objects, such as feathers, as attachment sites lead automatically to the increase of buoyancy. In any case the total positive buoyancy of the float must compensate the weight of the attached animals.

**Reduced pressure experiments**

At atmospheric pressure and at underpressure of 700 mbar, the float buoyancy was unaffected. This means that the walls of the bubbles in the cement could sustain at least 700 mbar. It was observed that at an ambient pressure below 400 mbar the cement burst and the
gas escaped from the bubbles. The float was now open to the outside, water filled the voids
and the floats began to sink. The fact that the floats sank due to structural failure of the
cement is an indirect proof that the intact cement float is a porous system closed to the
surrounding water.

Conclusions

The prerequisite for the use of the *Dosima fascicularis* cement as a float is its positive
buoyancy depending on the balance between the volume of the float and the volume of the
gas within the bubbles. The mass and the volume of the float depend on the size of the
attached animals and not on their number. The water content of the float is >90% and the gas
volume is on average 18.5%. Although the float is porous, it is crucial that it is sealed from
the surrounding water. In addition, a kind of rind gives mechanical stability to the float and
forms a barrier to the environment.

All lepadomorph barnacles are floating and the function of their cement is adhesion as in
other barnacles. In *D. fascicularis* the cement has a double function: Initially it is adhesion
and in addition it is floating independent of any floating substratum. The cement of *D.
fascicularis* is an interesting example of how the adhesive material can change its function
due to a slight modification of the structure. The use of the cement as a float at the water
surface allows the essentially sessile adult animal secondary mobility. By that *Dosima* can
extend its habitat and occupy new ecological niches.

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References


[22] Zheden, V., Klepal, W., Gorb, S. Kovalev, A. *submitted* Mechanical properties of the cement of the stalked barnacle *Dosima fascicularis* (Cirripedia, Crustacea). *Interface Focus.*


Table caption

Table 1. Summary of the physical values measured in 10 floats to which a varying number of animals were attached.

<table>
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<th>float number</th>
<th>animals attached</th>
<th>wet mass of the float (g)</th>
<th>volume of the float (ml)</th>
<th>volume of the gas (ml)</th>
<th>buoyant force (mN)</th>
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Figure captions

Figure 1. (a) Single *Dosima fascicularis* washed ashore. Scale bar: 1 cm. (b) Several animals share one cement float. Scale bar: 1 cm. (c) Experimental setup for measuring the buoyant force. The cement ball (c) floating in sea water was pushed under the water surface with a thin wire-frame (w) attached to a force transducer (ft). The red arrow indicates the movement of the platform.

Figure 2. (a) Stereo microscopic image of a razor blade section through a cement float (partly torn). The cement formed concentric layers around the stalk (s) and the alga (a). Scale bar: 2 mm. (b) Scanning electron micrograph of a cross section of a cement float. Large bubbles (b) were mainly in the inner region of the float, whereas in the outer region forming a kind of rind small, round bubbles (*) were dominant. Scale bar: 1 mm.
Figure 3. Correlation between the volume of the cement float and its wet mass. The data were fitted by random bootstrap for linear regression: \( y = -0.57 + 1.43x \), \( R^2 = 0.96 \), \( P < 0.001 \); \( R^2 \) = coefficient of determination.

Figure 4. (a) The histogram shows the wet mass of five floats, which consists of the dry portion (black) and the water content (grey) of the cement. Floats 4 and 8 were air dried, floats 3, 5 and 9 were oven dried. (b) The histogram shows the wet mass (grey), the dry mass (black) and the mass of the dried floats after three days kept in water (dark grey). After three days the dried floats sometimes took up more than 20% of water but they did not return to their original mass.
**Figure 5.** (a) Correlation between the volume of the gas inside the cement and the volume of the float. The data were fitted by random bootstrap for linear regression: $y = -0.05 + 0.2x$, $R^2 = 0.65$, $P = 0.018$. (b) Graph of a typical buoyant force measurement. The buoyant force of a cement float was defined as an average force in the area between the two grey lines (in this case 9.05 mN). (c) Correlation between the volume of the gas inside the float and the buoyant force: $y = -0.19 + 9.6x$, $R^2 = 0.99$, $P < 0.001$. 
Figure 6. (a) Differences between the buoyant force of the cement floats at atmospheric pressure (black) and after the exposure to reduced pressure: 700 mbar (grey) and 30 mbar (grey with red frame). (b) Cement float in a beaker filled with sea water in a vacuum desiccator at 200 mbar. Gas bubbles leaked out of the burst cement (arrows).
VI. Discussion

For the first time, this thesis characterises comprehensively the cement of *Dosima fascicularis*. The topics morphology, biochemistry and mechanical properties are highlighted. This animal is the most specialized pleustonic goose barnacle (Cheng & Lewin 1976), unique in producing a cement float which gives positive buoyancy to the animal and enables it to drift autonomously at the water surface (Thiel & Gutow 2005).

The first manuscript (Zheden et al. 2012) gives the first ever detailed morphological description of the cement apparatus of *D. fascicularis*. The morphology of the cement glands is comparable to those of *Balanus hameri* and *Lepas anatifera*. In these species the organelles are distributed evenly throughout the cytoplasm of the gland cells (Lacombe & Liguori 1969; Walker 1970; Jonker et al. 2012) which is considered an original condition. Within the gland cell of more derived barnacles (e.g. *B. tintinnabulum*, *B. psittacus*, *Elminius modestus*) a secretory and a storage region can be distinguished (Lacombe & Liguori 1969; Lacombe 1970; Walker 1970). The shape and the size of the *Dosima* cement gland cells change with the developmental stage of the barnacle. In *Semibalanus balanoides* this was interpreted as a primitive condition (Lacombe 1970). The nucleus of the gland cells alters from round to lobed and the number of nucleoli increases during gland development. In more advanced barnacles all stages of gland cell development can be observed at the same time (Lacombe 1970).

*D. fascicularis* is the only barnacle known so far which produces its own float. The highly specialized capacity to use the cement not only as an adhesive but also as float is unique amongst barnacles and considered a derived characteristic. The gas inside the bubbles of the cement is presumably CO₂, a metabolic product. We assume that CO₂ diffuses from the hemolymph into the lumen of the cement canal through the cells lining the collector and secondary canals. From there it is transported to the outside together with the cement through the cuticle lined principal canal.
The second manuscript (Zheden et al. 2014) deals with the biochemistry of the cement of *D. fascicularis*. Our data reveal that the *Dosima* cement consists of 84% proteins. The dominance of proteins is a general characteristic of barnacle cement (Barnes & Blackstock 1974, 1976; Naldrett & Kaplan 1997; Kamino 2006) and is also known from other marine adhesives (Stewart et al. 2004; Silverman & Roberto 2007). We identify protein bands with a molecular weight between 47.4 and 250.0 kDa which have their isoelectric points in the acidic range. In agreement with acorn barnacles high amounts of hydrophobic amino acids are in the *Dosima* cement. These render the cement matrix insoluble (Naldrett & Kaplan 1997; Kamino et al. 2012). So far the sequence analysis reveals that the *Dosima* cement consists of a *de novo* set of proteins with low homologies to other barnacle cement proteins. In the cement of *Dosima* we find sulphur but no disulphide bonds, which are commonly present in the adhesive of acorn barnacles to stabilize the protein complex and contribute to its insolubility (Naldrett 1993; Kamino et al. 2000). Polysaccharides are also detected in the *Dosima* cement. This could indicate that sulphated polysaccharides might occur in the *Dosima* cement as in the adhesive of the sandcastle worm *Phragmatopoma californica* (Wang & Stewart 2013).

The third manuscript (Zheden et al. submitted-a) is the first study of mechanical properties of the cement of a stalked barnacle. It comprises the results of the first mechanical tests of the special cement of *D. fascicularis*. We show that the values of the elastic modulus, hardness and tenacity of the *Dosima* cement are considerably lower than in the rigid cement of acorn barnacles (Watermann et al. 1997; Sun et al. 2004; Ramsay et al. 2008; Sullan et al. 2009; Sangeetha & Kumar 2011). Our results reveal that the *Dosima* cement is one of the softest biological adhesives. Its elastic modulus (5-20 kPa) lies in the same range as that of the tube foot disc of echinoderms (Santos et al. 2005) or the adhesive pads of ensiferan insects (Perez Goodwyn et al. 2006). Two regions can be distinguished within the cement float: a softer interior and a compact harder outer zone. The main function of the inner region, which contains large gas-filled bubbles, is to give buoyancy to the animal. The outer cement layers
form a kind of rind with small gas-bubbles. This rind acts as protection towards the environment and gives mechanical stability. Similar structural and mechanical characteristics are also found in the adhesives of Phragmatopoma californica and Mytilus edulis, where the densest material is at the interface to the surrounding water (Benedict & Waite 1986; Stewart et al. 2004).

Emphasis of the fourth manuscript (Zhen et al. submitted) lies on the buoyancy of the cement float. D. fascicularis is the only barnacle which secretes a high amount of foam-like cement and uses it as a float (Boëtius 1952-53). Apart from the individual use it can form free-floating colonies attached to a central float (Ryan & Branch 2012). The size of the attached animals and not their number determine the volume and the mass of the float. The gas content within the bubbles of the cement is crucial for the positive buoyancy of the whole animal. We detect an average gas volume of 18.5% in the float. The water content of the cement is more than 90%. Our pressure experiments show that the cement float is a porous system closed to the surrounding water. When the float is damaged, water replaces the gas and the float sinks.

In summary the cement apparatus of Dosima fascicularis resembles that of other barnacles. The cement, one of the softest biological adhesives, is highly specialized and differs from that of other barnacles in its structure, chemical composition and mechanical properties. In comparison with the cement of all other barnacles investigated so far the Dosima cement is an interesting example of how an adhesive material changes its function due to a slight modification of the structure. The inclusion of gas-filled bubbles and the resulting capacity to drift allows extension of the animal’s habitat and the occupation of new ecological niches.

Investigations on the morphology, chemical composition and mechanical properties of biological adhesives will, no doubt, inspire the development of synthetic bio-adhesives with applications in medicine and industry (Smith & Callow 2006; von Byern & Grunwald 2010;
Favi et al. 2014). These bio-inspired adhesives are promising because of their non-toxicity, quick binding and their high strength (Favi et al. 2014). Barnacle cement can serve as a powerful dental adhesive (Ang et al. 2011) and can lead to the development of environmentally benign antifouling marine coatings (Callow & Callow 2011). Dosima cement, in particular, can be used to develop scaffolds for tissue growth and wound healing (Cooper & Kennedy 2010; Kennedy & Cooper 2013). Due to its shock-absorbing properties a possible application in orthopedics seems feasible.

References


VII. Abstract

Cirripedia Thoracica are sessile marine Crustacea. They comprise acorn and stalked barnacles, all of which attach to the substratum with an adhesive (cement) produced by cement glands. The stalked barnacle *Dosima fascicularis* is unique amongst the Cirripedia, because it uses its adhesive not only for attachment but also as a float. The animal produces an excess amount of proteinaceous, foam-like cement with enclosed gas filled bubbles. With the float the normally sessile animal can drift autonomously at the water surface.

In this thesis the morphology of the cement apparatus and the cement of *Dosima fascicularis* was analyzed in detail for the first time. The pathway of the cement was followed from its production in the cement gland cells to its secretion through pores at the base of the stalk where it polymerizes in the sea water.

Further the biochemistry of the *Dosima* cement was investigated. The protein and carbohydrate content of the cement as well as the amino acids and chemical elements were analyzed. In its chemical composition the *Dosima* cement is similar to that of other barnacles, but it also shows interesting variations.

In this thesis mechanical properties like elastic modulus, hardness and tensile stress of the cement float were tested for the first time. The values of these mechanical properties are considerably lower in the very soft and visco-elastic *Dosima* cement than in the rigid cement of other barnacles. Of special interest was the buoyancy of the float, which depends on the balance between the volume of the float and the volume of the gas in the cement.

The studies on the morphology, the chemical composition and the mechanical properties of the *Dosima* cement are of particular interest because of the possible application of this biological adhesive in medicine and industry.
VIII. Zusammenfassung

Cirripedia Thoracica sind marine, festsitzende Krebs (Crustacea), welche Seepocken und Entenmuscheln beinhalten. Diese Tiere setzen sich mit Hilfe eines überwiegend proteinhaltigen Adhäsivs (Zement) am Substrat fest. Die Entenmuschel *Dosima fascicularis* ist einzigartig unter den Cirripedia, da sie ihren Zement nicht nur zum Festsetzen sondern auch als Floß verwendet. Dieses Tier produziert eine große Menge an schaumigen Zement der mit Gasblasen gefüllt ist. Mit diesem Zementfloß kann das ursprünglich festsitzende Tier autonom an der Wasseroberfläche treiben.

In dieser Doktorarbeit wurde zum ersten Mal detailliert die Morphologie des Zementapparats und des Zements von *Dosima fascicularis* untersucht. Es wurde der Weg des Zements von seinem Entstehungsort in den Zementdrüsenzellen bis zu seiner Sekretion über Poren an der Stielbasis des Tieres verfolgt.

Weiter wurde die chemische Zusammensetzung des Zements analysiert, vor allem der Protein- und Kohlehydratanteil, die involvierten Aminosäuren sowie die chemischen Elemente. Die chemische Zusammensetzung des *Dosima* Zements ist ähnlich der anderer Cirripedia, weist aber einige interessante Unterschiede auf.


IX. Acknowledgments

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X. Curriculum vitae

Personal Data
Name: Mag. Vanessa Zheden
Place of birth: Friesach (Carinthia)
Nationality: Austrian

Studies
since 2009
PhD thesis supervised by Prof. Dr. Waltraud Klepal:
Characterization of the cement of the stalked barnacle Dosima
fascicularis: Morphology, biochemistry and mechanical properties
03/07 – 11/08
Diploma thesis supervised by Prof. Dr. Waltraud Klepal:
Cuticle structures on different body regions of Semibalanus balanoides
(L.) (Thoracica, Cirripedia) with special emphasis of mature and
degenerating penis
since 2003
Zoology, University of Vienna
10/99 – 05/03
Biology, University of Vienna

Work experience
08/09 – 12/13
Employee and collaborator within the Austrian Science Fund (FWF)-
project to Prof. Dr. Klepal on the morphology, biochemistry and
mechanical properties of the cement apparatus in Dosima fascicularis at
the Core Facility Cell Imaging and Ultrastructure Research (CIUS),
University of Vienna
10/07 – 07/09
Tutor for “Submicroscopical anatomy and preparatory techniques in
electronmicroscopy” at CIUS
01/04 – 06/07
Preparation and identification of bone fragments from different
evacuation sites at the archaeozoological collection of the Natural
History Museum Vienna by order of the Austrian national heritage
agency (Bundesdenkmalamt)

Scholarships
08/14 – 10/14
PhD Completion Grant of the Faculty of Life Sciences, University of
Vienna
30.07. – 23.08.13
Short-term scientific mission (COST-action TD 0906) at the Christian-
Albrechts-Universität Kiel (Germany), in the group of Prof. Dr.
Stanislav Gorb: to test the mechanical properties of the cement of
Dosima fascicularis
03/11 – 07/11
DAAD-Scholarship at the Fraunhofer Institute for Manufacturing
Technology and Advanced Materials IFAM, Bremen (Germany), in the
group of Dr. Ingo Grunwald: to analyse biochemically the cement of
Dosima fascicularis

Memberships
ASEM – Austrian Society for Electron Microscopy
COST-Action TD0906: Biological adhesives: from biology to biomimetics
Publications


Zheden V., Klepal W., Gorb S.N., Kovalev A. Mechanical properties of the cement of the stalked barnacle Dosima fascicularis (Cirripedia, Crustacea). Submitted to Interface Focus.


Book chapters


Some popular scientific articles

“Superkleber der Meere“ in the online newspaper of the University of Vienna, August 2010

Conferences

25.08. – 30.08.13  Microscopy Conference MC 2013 in Regensburg (Germany)
Talk: “The cement float: morphology, biochemistry and mechanical properties of the barnacle *Dosima fascicularis* (Crustacea, Cirripedia Thoracica)”

26.05. – 27.05.12  2nd ASEM-Workshop Advanced Electron Microscopy in Salzburg (Austria)
Talk: “The buoy barnacle *Dosima fascicularis* with its special cement”

05.09. – 07.09.11  International Conference: Marine Resources and Beyond in Bremerhaven (Germany)
Talk: “Characterization of the cement formation in *Dosima fascicularis* (Crustacea, Cirripedia)”

28.08. – 02.09.11  Microscopy Conference MC 2011 in Kiel (Germany)
Talk: “The cement apparatus of *Dosima fascicularis* (Ellis and Solander, 1786) (Crustacea, Cirripedia Thoracica)”

18.05. – 20.05.11  Biological and Biomimetic Adhesives in Mons (Belgium)
Talk: “*Dosima fascicularis* a barnacle which floats rather than sticks”

21.10. – 24.10.10  3. Graduiertenforum der Fachgruppe Morphologie in Vienna (Austria)
Talk: “Morphologie und Ultrastruktur des Zementapparats von *Dosima fascicularis* (Crustacea, Cirripedia)”

30.08. – 04.09.09  Microscopy Conference MC 2009 in Graz (Austria)
Poster: “Temporary adaptation of the cuticle in *Semibalanus balanoides* (Linnaeus, 1767) (Crustacea, Cirripedia Thoracica)”