The natural adhesive of the goose barnacle *Lepas anatifera*:

The functional morphology and chemistry of the adhesive gland and an investigation of the adhesive proteins

by

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September 2013

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Declaration

I hereby declare that this thesis is my own original research work, conducted under the supervision of Dr Anne Marie Power. This work has not previously been submitted for the award of any other degree or diploma.

The work described in chapter 2, the collection and cultivation of goose barnacles, was conducted alongside another student and described in: McEvilly, P. (2011). *A look at some components of the adhesive of the pedunculate barnacles Dosima fascicularis, Lepas anatifera, Conchoderma auritum, Lepas pectinata and Pollicipes pollicipes.* (Research Masters), National University of Ireland, Galway, Galway. This work was divided equally between myself and Paul McEvilly.

Chapter 3 has previously been published as: Jonker, J.-L., von Byern, J., Flammang, P., Klepal, W., & Power, A. M. (2012). Unusual adhesive production system in the barnacle *Lepas anatifera*: An ultrastructural and histochemical investigation. *Journal of Morphology, 273*(12), 1377-1391. This work was performed and written by myself with co-authors acting as teachers and supervisors of histology and histochemistry (J. von Byern), immunohistochemistry (P. Flammang) and ultrastructure (W. Klepal).

Chapter 4 was conducted with the help and supervision of Dr Liam Morrison, for his expertise in scanning electron microscopy, energy dispersive spectrometry and Raman spectroscopy.

Acknowledgements

First and foremost, I want to thank my supervisor Anne Marie Power for providing me with the opportunity to travel to Ireland and complete a PhD at NUI Galway. She and the rest of the Zoology department of NUI Galway, especially the technical staff, provided invaluable support throughout my time there.

Second, I would like to thank my friend and colleague Paul McEvilly for being my constant companion throughout the first half of my PhD and continuing to provide support and optimism from afar. Further thanks go out to all of the friends I made during the course of my PhD, many of whom were also my teachers for various aspects of this multidisciplinary work; you are too numerous to name but I truly could not have made it to the end without you all so thank you.

Many colleagues provided me with vital skills and advice over my years at NUI Galway, choosing to give up their time and energy to teach me and help find solutions when things went wrong. Thanks to Professor Waltraud Klepal, Dr Janek von Byern and Vanessa Zheden for teaching and looking after me so well in Vienna, to Ingo Grunwald and his teaching assistants for my time in Bremen and to Professor Patrick Flammang and Dr Elise Hennebert for my short time in Mons.

Many thanks to Pearce Lalor and Mark Canning of the NUI Galway Anatomy department, for taking such an interest in my work even though they didn't have to, and the rest of the Anatomy department for allowing me to work there and helping me when I needed it. Dr Liam Morrison chose to take an interest in my studies and was full of enthusiasm and ideas, giving me his time and skills and providing me with further contacts, all of which gave my PhD a necessary boost. Dr Florence Abram of the NUI Galway Microbiology department not only chose to teach me, but gave me support and friendship and I am extremely grateful for her help.

Finally, to the all the people out there, scattered across various countries, that gave me advice, information, support, friendship, or just a laugh, I would like to thank you for being a part of this huge task that has been my life for the last four years.

Abstract

The adhesive and adhesive glands of the stalked barnacle *Lepas anatifera* were investigated using a wide range of techniques to characterise them and compare them to distantly related acorn barnacles, as well as other adhesive models such as mussels, tubeworms and echinoderms. Live samples of *L. anatifera* were collected from Irish coastal waters and maintained in a laboratory aquarium. Histochemical and immunological analyses showed that the adhesive production system in barnacles was unique compared to other adhesive animal models and showed that, within the body, the adhesive is slightly acidic, contains some carbohydrate and does not contain L-3.4-dihydroxyphenylalanine or phosphorylated serines. Ultrastructural analyses revealed a series of single celled adhesive glands, drained by ever-larger canals. The first, the intracellular canal, is immediately adjacent to the gland cell cytoplasm and responsible for transferring the secretion granules across the cell membrane through apocrine and merocrine processes. Once they had been released from the gland cell cytoplasm, the adhesive components were completely unbound by granular membranes as they made their way through the adhesive drainage system. These observations raise questions about how premature hardening of the adhesive is prevented.

Because of their relatively large size, it was possible to elementally map the gland cells for the first time using SEM-EDS. These analyses showed that the gland cells had a far less varied elemental composition than the adhesive. A wider range of elements than has been recorded previously was observed in the adhesive, although some of these were probably environmental contaminants. Elements were sometimes variable, for example, phosphorus was sometimes present in the gland but mostly absent from the adhesive. Sulfur was absent from the gland but always present in the adhesive. FTIR and Raman spectroscopy indicated that the sulfur content of the adhesive was not present in disulfide form. Spectroscopic analyses confirmed the absence of phosphorylated proteins and protein-metal interactions. $CaCO_3$ involvement in barnacle adhesion was also ruled out but Raman spectroscopy showed a signal consistent with tyrosine in the adhesive. There were marked differences in Raman spectra between stalked and acorn species.

The hardened adhesive was solubilised most successfully by increasing the amount of urea/thiourea buffer prior to 1D SDS-PAGE. Approximately 12 bands were observed ranging in size from less than 20 kD to 200 kD. Repeating 1D SDS-PAGE showed consistent bands at 30, 70, 80, 90, 110 kD. Mass spectrometry of these protein bands showed no significant similarity to any known proteins, including published barnacle adhesive proteins. *De novo* sequencing of adhesive protein bands produced seventy-eight peptide fragments of up to 16 amino acids in length. The longest *de novo* sequences (more than 11 amino acids) were subjected to multiple BLAST analyses and compared to translated cDNA open reading frame sequences from a transcriptome of *Amphibalanus amphitrite* and an unpublished database for *Tetraclita*, but these returned no matches. Several of the *de novo* sequences were repeated across several of the bands that were analysed, indicating possible multiple variants of the same protein. There was some limited similarity in the protein size bands and peptide sequences of *L. anatifera* and related species *Dosima fascicularis*, but less similarity to size ranges of acorn barnacle proteins. All attempts to amplify adhesive protein genes using PCR with primers based on *de novo* peptide sequences were unsuccessful.

Immunohistochemical assays using polyclonal antibodies raised against acorn barnacle adhesive proteins resulted in positive reactions in *L. anatifera* adhesive gland tissue for two out of the three proteins tested, suggesting that some homology exists between stalked and acorn barnacle adhesive. However, extensive attempts to isolate adhesive protein genes from *L. anatifera* cDNA using primers based on acorn barnacle adhesive protein gene alignments, as well as acorn and stalked barnacle cDNA sequences for adhesive genes, were unsuccessful. Homology between acorn barnacles and *Pollicipes pollicipes* sequences ranged from 26% to 36% depending on the gene. Overall, there were strong distinctions between the adhesives of distantly related (different taxonomic order) barnacle species. The overall picture which emerges is one where the functional homology of barnacle proteins does not strictly depend on the characteristics of size, pI or primary sequence similarity.

1 General introduction

1.1 Underwater adhesion

"For a large number of problems there will be some animal on which it can be most conveniently studied"

(Krogh, 1929)

1.1.1 Bioadhesion

In biology adhesion is a matter of course, and for many organisms it is a way of life. At the most basic level, adhesion is essential for maintaining multicellular structure and relies upon cell-surface proteins that bind to other cell-surface proteins. To move beyond the molecular bonding of single cells, adhesion is widely utilised by complex multicellular organisms. Adhesion can be completely mechanical, such as the interlocking structures that cause some plant seeds to cling to passing animals (Gorb & Gorb, 2002), or can make use of molecular interactions, such as ionic bonds, covalent bonds or van-der-Waals forces. Geckos use van-der-Waals forces to adhere to a wide range of surfaces; microscopic projections cover the hair-like setae on their footpads, creating a huge surface area that is in intimate contact with a surface (Autumn et al., 2000). Chemical bonding as a form of bioadhesion is generally achieved by the secretion of a substance that has the capability of bonding to a substrate. Examples of secreted adhesive substances can be found amongst bacteria, plants and animals, however it is animal adhesives that are often immediately called to mind. Spiders spin sticky webs, caterpillars make cocoons, snails stick to walls and barnacles stick to rocks and ships (and everything else imaginable in the sea). Some adhesives are temporary and allow movement, such as snail mucus, while others are permanent and very strong, such as the adhesives of marine animals like mussels and barnacles.

Bioadhesion is an interesting phenomenon in itself, however most attention placed on bioadhesives concerns how they can be utilised for our own human needs. In the past the adhesive properties of natural substances have been utilised directly, as in the case of vegetable and animal glues, and have also been mimicked. Velcro is a well-known mimic of natural mechanical adhesion and was inspired by the hooked barbs of plant seeds (De Mestral, 1955). Other examples include adhesives used in surgery that utilise the mammalian body's own clotting mechanisms, fibrin and thrombin. Fibrin sealants

are currently used to aid blood clotting and seal surgical wounds in certain tissue types (Spotnitz, 2010). Concerns about relatively weak bond strength of fibrin and cross-infections from bovine sources of this protein have meant that the search has continued for novel sources of adhesives, for example, from invertebrates. More recently a substance known as chitosan has been marketed as a biomedical adhesive product: this polysaccharide extracted from the shells of shrimps and other crustaceans has blood-clotting properties and is currently used in bandages and other haemostatic (blood clotting) agents (Kumar, 2000).

These medical applications show bioadhesives being used as biomaterials, which are any (natural or synthetic) materials interacting with living tissue (Temenoff & Mikos, 2008). In the field of biomaterials the term bioadhesives may be considered to mean any adhesive that is applied to living tissue. However, for the purpose of the current thesis the term bioadhesion and bioadhesive will only be used to refer to adhesive substances produced by living organisms.

1.1.2 Bioadhesion underwater

From a technological point of view, adhesion and water do not go well together. Water on a surface will compete with the molecules of an adhesive to form bonds with a substrate and can permeate the adhesive and displace adhesive bonds that are already in place (Smith & Callow, 2006). Man-made underwater adhesives currently available are few and far between; cyanoacrylates (such as super-glue) in general can maintain a bond underwater, however underwater application is difficult as the liquid adhesive sets immediately upon contact with water (Cloete & Focke, 2010). Epoxy adhesives are also available for underwater application; while some underwater epoxy adhesives have an adhesive capacity nearly equivalent to dry setting adhesives (Kim et al., 2009), others appeared to fail faster than adhesive joints created in a dry environment (Frantzis, 2008). It is clear that man continues to struggle to create a synthetic adhesive that can be used in wet conditions (including during surgery), however nature has shown time and time again that strong adhesion underwater is possible. Bioadhesion has arisen in a wide variety of animals from various different phylums, both in freshwater and saltwater.

The study of permanent adhesives produced by animals such as mussels, tubeworms and barnacles has a long history. Initially thought to be a simple problem with a simple solution, it was eventually recognised to be a highly complex biological phenomenon involving many proteins and associated ions. To adhere underwater an animal must produce a substance that can remove weak boundary layers on a substrate, spread over a surface, form strong bonds with the surface and then cure or cross-link to stabilise the bond (Waite, 1987).

While the complete understanding of adhesion used by mussels, tubeworms and barnacles remains unknown, researchers came across a vital functional group in early investigations of mussel adhesive, which has provided inspiration ever since. L-3,4-dihydroxyphenylalanine (DOPA) was found in the byssal adhesive plaques of *Mytilus edulis* and can be adsorbed to many surfaces (Waite & Tanzer, 1981). DOPA has also been shown to be present in the cement of the tubeworm *Phragmatopoma californica* (Waite et al., 1992) and *Sabellaria alveolata* (Becker et al., 2012). Another common factor of many underwater adhesives is phosphorylated serine (pSer), which is present in the adhesive plaque proteins of mussels (Waite & Qin, 2001), the cement of tubeworms (Zhao et al., 2005), the silk of the caddisfly larvae (Stewart & Wang, 2010) and the cuvierian tubules of sea-cucumbers (Flammang et al., 2009).

The presence of oppositely charged acidic pSer-rich and basic DOPA-rich proteins in the tubeworm adhesive led to a theory that the adhesive functioned through complex coacervation (liquid-liquid phase separation) (Stewart et al., 2004). This theory describes the adhesive as a liquid separated into non-mixing phases; the proteins and associated ions combine to form a dense, neutral phase (the coacervate), leaving the surrounding substance (equilibrium phase) depleted and of a different density (Waite et al., 2005). In the tubeworm model, the release of the adhesive into the saltwater environment disrupts the neutral charge of the coacervate phase, causing the proteins and ions in that phase to interact in new ways, forming cross-links and hardening (Stewart et al., 2004). This theory has been successful in providing inspiration for a new underwater bioadhesive mimic (Shao et al., 2009), yet recent studies indicate that complex coacervation is not responsible for tubeworm adhesion (Wang & Stewart, 2012).

1.2 Barnacle adhesion

"A new problem to solve: to attach a crustacean permanently to a foreign body; no one could tell by what singular and novel means this could be effected"

(Darwin, 1851)

1.2.1 Barnacles

Barnacles are sessile, filter feeding crustaceans with mineralised plates (shells). The subclass Cirripedia is generally divided into two groups based on body shape, the stalked barnacles (the stalk is also known as the peduncle) and the acorn barnacles. The range of species that will be discussed throughout this thesis belong to three different orders (Figure 1.1). The Lepadiformes are stalked barnacles with five capitular (body) plates and no peduncular scales. The Sessilia are the acorn barnacles and have no stalk, while the Scalpelliformes are stalked barnacles with additional capitular plates and peduncular scales. Though they possess a stalk, molecular phylogenies have shown the Scalpelliformes to be a sister group to the Sessilia, with Lepadiformes being placed outside of this clade (Harris et al., 2000; Linse et al., 2013; Perez-Losada et al., 2004). The development of all barnacles proceeds through a series of naupliar larvae, similar to other crustaceans, but has a unique final larval stage: the cyprid. The cyprid searches for a suitable substrate to attach to; it uses a temporary adhesive when exploring a surface and releases a permanent adhesive when a suitable surface has been found. The cyprid then undergoes a moult which transforms it into a juvenile barnacle. Throughout its life the barnacle adds to the adhesive plaque to reinforce its attachment (Anderson, 1994).

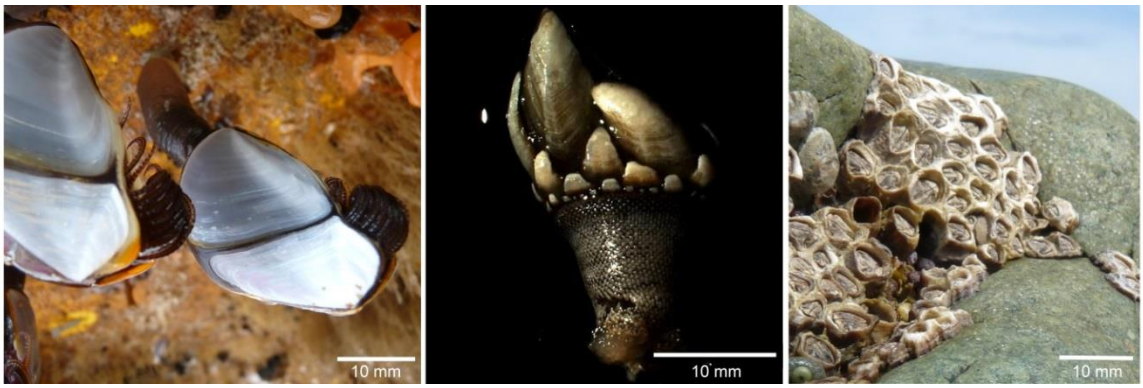

Figure 1.1: Three different types of barnacle: A) *L. anatifera*, order Lepadiformes; B) *P. pollicipes*, order Scalpelliformes, image by Paul McEvilly; C) acorn barnacle species, order Sessilia.

Lepas anatifera, the primary species of interest in this thesis, is a large barnacle with a flexible peduncle (stalk) covered in a tough cuticle. It is a neustonic species, inhabiting the surface of the ocean, and settling gregariously to floating objects. *L. anatifera* can attach to a wide range of substrates, including wood, plastic, glass, rope, metal, seaweed, other animals and anti-fouling coated surfaces (such as data buoys); this is in contrast to some of the more specialised species, for example *Chelonibia* species attach to the skin of turtles and other marine animals. Substrate generalists such as *L. anatifera* form a significant part of fouling communities on man-made objects in the sea and can be a costly problem for governments and businesses; to take the shipping industry as an example, it has been shown that heavy calcareous fouling could lower a vessel's efficiency by 86% (Schultz, 2007). Extensive efforts have been made to combat fouling by barnacles by studying their settlement and their attachment to various surfaces, including textured and low surface-energy anti-fouling surfaces, however such studies have been carried out in just a few acorn barnacle species, such as *Amphibalanus amphitrite* (e.g., Aldred et al., 2010; Chen et al., 2011; Dreanno et al., 2006; Holm et al., 2009). Large stalked barnacles have been investigated to a far lesser degree than many acorn barnacles, which is most likely due to two reasons: the neustonic lifestyle of these species impedes easy collection of specimens and the larval development is much slower and has not been successfully achieved in a laboratory environment.

1.2.2 The barnacle adhesive apparatus

One of the earliest and most in depth studies of barnacle morphology and taxonomy gave a detailed account of the morphology of many different species of Lepadiformes, Scalpelliformes and Sessilia, including an interpretation of the adhesive apparatus (Darwin, 1851, 1854). This study was conducted without the help of modern microtomy and histology techniques, the lack of which led Darwin to mistake certain structures, yet upon examination of the text and diagrams, one can see that he did actually describe what we now know to be the adhesive apparatus. Darwin appears to have accurately located the main ducts (later termed principal canals) that transport the adhesive to the substrate, but did not succeed in tracing these to the adhesive glands. Instead, he has described an enlargement of the main canal to be the gland. He then went on to describe how the ovary included ovarian tubes, connected to developing ova, which were continuous with the gland and main canal. These 'ova' were later confirmed by several

independent researchers, such as Krohn (1859) and Koehler (1889), to in fact be the adhesive glands and canals. These later studies described the presence of many unicellular gland cells, connected to a network of branched canals, some of which were lined by a thick cuticle.

A surge in barnacle adhesive research appeared a century later in response to concerns over biofouling and interest in natural adhesives (e.g., Cheung & Nigrelli, 1972; Fyhn & Costlow, 1976; Lacombe & Liguori, 1969; Walker, 1970). These later studies provided a basic understanding of the apparatus that produces the adhesive, which is still relied on by many current researchers: the adhesive glands are large, isolated secretory cells that are thought to each produce all of the elements that make up the adhesive. The large, principal canals that deliver glue to the substratum connect to the adhesive glands via a series of smaller, secondary canals, which branch to form collecting canals. The small collecting canals branch further, where they meet the gland cell, to form intracellular canals. Walker (1970, 1978) examined the ultrastructure of the adhesive apparatus of several different acorn barnacle genera and in some two markedly different regions of cytoplasm were observed. One was defined as the storage pole, consisting of large accumulations of secretory granules filled with moderately dense, diffuse material; the other was defined as the synthesis pole of the cell and had a predominance of rough endoplasmic reticulum, loose ribosomes, mitochondria and Golgi bodies. In most species, a dense ‘cuticle’ lining was observed on the inside surface of the principal canal.

The barnacle cyprid larva is responsible for settlement and initially produces a temporary adhesive that it uses to explore a substrate (Walker & Yule, 1984). When a suitable substrate is found a permanent adhesive is secreted which contains phenols and polyphenol oxidase, indicating a quinone cross-linking process akin to the adhesive mechanism of mussel byssus (Walker, 1971). The cyprid adhesive gland contains two secretory cell types and it has long been thought that the cyprid adhesive gland breaks down completely at metamorphosis and is not related to the adult adhesive gland (Cheung & Nigrelli, 1972). New evidence suggests that the cyprid and adult adhesives do share some common features; homologues of the adult barnacle adhesive protein cp-20k have been located in both of the cyprid adhesive secretory cell types (He et al.,

2013), while it has also been recently suggested that phenols, though long dismissed, may play a role in adult barnacle adhesive after all (Burden et al., 2012).

1.2.3 Barnacle adhesive

Early biochemical studies showed that the adhesive was primarily composed of protein (more than 90%) (Barnes & Blackstock, 1974, 1976; Walker, 1972), however further investigations of the adhesive proteins were impeded by the insolubility of the adhesive. An early report described partially solubilising the adhesive of three acorn barnacles, which was then separated into several protein bands, however most bands appeared inconsistently and the adhesive often repolymerised before it could be separated (Naldrett, 1993). Kamino et al. (2000; 1996; 1998) described four proteins from barnacle adhesive and reported the presence of five more. The first protein that was fully sequenced by this group was cp-100k, a hydrophobic protein with a short alternating pattern of hydrophobic and hydrophilic residues (Kamino et al., 2000). Cp-20k is a small, hydrophilic protein with an abundance of cysteine residues that form intramolecular disulfide bonds and are vital for correct protein folding (Kamino, 2001; Suzuki et al., 2006). A recombinant form of cp-20k showed that this protein was specifically adsorbed onto calcite (Mori et al., 2007). Cp-19k is another small, hydrophilic protein with a biased amino acid composition, with high concentrations of alanine, serine, glycine, threonine, valine and lysine. The recombinant form of cp-19k was shown to be easily adsorbed to a range of different surfaces (Urushida et al., 2007). Homologues of cp-100k, cp-20k and cp-19k have been found in several different barnacle species from the order Sessilia (Kamino, 2008; Mori et al., 2007; Urushida et al., 2007). Cp-52k is the most recently characterised barnacle adhesive protein; it is a large hydrophobic protein consisting of four long sequence repeats that are connected through intramolecular disulfide bonds. Cp-52k is N-glycosylated and the only barnacle adhesive protein found to contain a post-translational modification so far (Kamino et al., 2012). Though not fully characterised, early reports indicate that cp-68k may also be glycosylated (Kamino & Shizuri, 1998).

1.2.4 Mechanism of adhesion

The molecular mechanisms by which barnacle adhesive functions are unique from those used by other commonly studied adhesive organisms. Clues from the biochemical

properties of the adhesive as a whole and the individual proteins has allowed some suggestions to be made regarding its adhesive and cohesive abilities but for the most part the barnacle adhesive complex remains a mystery.

The barnacle adhesive is so far the only known marine adhesive to be dominated by hydrophobic proteins, leading to the suggestion that hydrophobic interactions and hydrogen bonds are one of the important factors for the cohesion of the cured adhesive (Kamino et al., 2012; Naldrett, 1993). Cysteine residues in the barnacle proteins so far appear to be required for intramolecular bonds only, conferring shape to each protein, as opposed to intermolecular bonds cross-linking the different proteins together (Kamino et al., 2012; Suzuki et al., 2006). It has been suggested that as disulfide cross-links are not involved it is the shape of the large proteins that is important for self-assembly and cohesion (Kamino et al., 2012). A lack of strong cross-linked structures could be advantageous as an open, unordered conformation would maximise interactions between potential binding sites and the substratum (Naldrett, 1993). It has also been suggested that some of the structural integrity of the cured adhesive is provided by proteins in the form of cross-β-sheet fibres, like amyloid protein aggregates (Barlow et al., 2010; Kamino et al., 2000; Sullan et al., 2009). Cross-β-sheet formations could lend insolubility and stability to the complex and encourage aggregation of components (Kamino et al., 2000). These folding structures might also impart cohesive strength via the large number of hydrogen bonds between cross-β-sheets that act as sacrificial bonds.

It is possible that the adhesive retains its fluidity within the body of the animal because the adhesive takes a long time to fully cure; the process that is begun within the adhesive glands of the barnacle is slow enough that the adhesive is released from the animal long before it has cured. A theory by Wiegemann (2005) is that the adhesive acts as a bio-colloid, in which the proteins aggregate in small groups and behave has micelles with the hydrophobic regions hidden within the hydrophilic regions. A protective emulsifying agent could be present around the micelles to prevent early aggregation, which could be disrupted by the change in environment when the adhesive is released from the animal, allowing the micelles to aggregate and interact with one another. This theory is somewhat similar to the complex coacervation theory of the tubeworm adhesive. However, this theory does not easily explain how the different

proteins interact with one another, nor how the small hydrophilic substrate-interactiving adhesive proteins fit into the biocolloid.

1.3 Adhesive biomaterials

When adhesives are used in medical and dentistry applications they can immediately be considered biomaterials and as such must meet certain requirements. Perhaps most important is that the substance must retain its adhesive properties in a wet environment, where the moisture is caused by bodily fluids. Just as importantly, adhesive biomaterials need to be safe and biocompatible, causing no adverse reactions, and must break down completely after healing so that no foreign body remains. An adhesive biomaterial must not hinder the process of healing and preferably will promote healing. It must also act locally and not migrate through the body (Reece et al., 2001).

Adhesives that are available for use on living tissue are limited and were initially developed as haemostatic agents, such as fibrin adhesives. Fibrin adhesives are not only used to aid blood-clotting but can also be used to seal leaks from tissues such as lungs, the lymphatic system and the blood-brain barrier, to control burn bleeding after removal of damaged skin, to adhere skin grafts to the underlying tissues and to seal minor lacerations (Reece et al., 2001; Spotnitz, 2010). Some forms of cyanoacrylates are also commonly used on living tissue; however cyanoacrylate cannot be broken down by the body and thus is only suitable for topical use. If the cyanoacrylate adhesive enters the wound a foreign body reaction may ensue, requiring further surgery (Dragu et al., 2009). The advantages of using cyanoacrylates over sutures are debated; some studies show application to be much faster than sutures, with better cosmetic results (Bozkurt & Saydam, 2008) while others disagree (Ong et al., 2002).

Several other adhesives are available for surgical applications, however their use is not yet widespread and their safety in various applications remains unknown. Gelatin-resorcinal-formaldehyde-gluteraldehyde (GRFG) and gelatin-resorcinal-gluteraldhyde (GRG, also called albumin-gluteraldehyde) combinations are adhesive and haemostatic but their safety is debatable. Currently used for aortic repair and to seal leaks during pulmonary surgery (Nomori et al., 1999; Reece et al., 2001), leaving the formaldehyde out of the adhesive appears to produce a smaller adverse reaction (Ennker et al., 1994),

however enough gluteraldehyde is released from the polymerised adhesive to have cytotoxic affects, indicating that these adhesives are not biocompatible and will not be suitable for use on more sensitive tissues. Another option currently in use is hydrogels (polyethylene glycol polymers, PEGs), which are water soluble and generally activated by light. While these tissue adhesives are bioadsorbable they take some time to apply and set (due to photoactivation) and take up to three months to break down (Reece et al., 2001).

Dentistry requires an entirely different sort of adhesive, as the surface being repaired is generally hard (enamel or dentin). Currently there are two primary substances used as adhesives and fillers in dentistry and orthodontics: glass isomers and composite resins (Nicholson, 2000; Tyas & Burrow, 2004). Glass isomers are generally used as fillers and blend in with the colour of the tooth; they adhere to the tooth via an acid/base reaction and are able to be used in situations where moisture cannot be completely removed, however they are not suitable for strength-bearing locations (Tyas & Burrow, 2004). Composite resins (also known as dentin-bonding adhesives) are similar to epoxy resins and contain harmful products such as formaldehyde, which are cytotoxic (Szep et al., 2002; Tyas & Burrow, 2004). While the adhesives are not meant to be applied to the soft tissues around the tooth, any accidental application can cause adverse reactions (Szep et al., 2002).

1.3.1 Bioadhesives as biomaterials

Much of the research into bioadhesion is directed at utilising or mimicking nature's adhesive mechanisms for our own good. Currently, a primary research directive is to create a better tissue adhesive for use in surgery, as the few currently available are very limited. While no biologically inspired tissue adhesives are yet available for human use, there are many options that are currently being tested on cell tissue cultures and animal models.

The near ubiquitous presence of DOPA in natural adhesives has given rise to a variety of adhesive biomaterials. PEG hydrogels have been modified with catechol (DOPA or the related dopamine) to create an adhesive that does not require photoactivation to set. They have been used successfully for islet-cell transplantation to treat diabetes (in the

rat model) and to seal foetal membranes after surgical incision (on human foetal membrane sections); results showed minimal inflammatory response and similar adhesion properties as fibrin glue (Bilic et al., 2010; Brubaker et al., 2010). DOPA has also been used to create a glue out of the natural polysaccharide chitosan, which was capable of adhering glass slides together and setting underwater (Yamada et al., 2000). The adhesive mussel foot-proteins (fp) have not only inspired tissue adhesive but been directly utilised after being extracted from the mussel foot: a product known as Cell-Tak is available to adhere cells to a substrate for cell and tissue culture applications (Dove and Sheridan, 1986). However, extracting bulk amounts of mussel foot-proteins is unfeasible and composition of the natural product is uncontrollable, thus recombinant forms have been created, using tyrosinase treatment to convert tyrosine residues into DOPA (Hwang et al., 2007a). To counteract the difficulty of creating a recombinant form of the highly adhesive fp-5, a hybrid of fp-1 and fp-5 was produced which had better cell adhesion than Cell-Tak and successfully promoted osteoblast proliferation when coated on a titanium implant surface (Hwang et al., 2007b; Hwang et al., 2010a).

Inspiration from another animal has given rise to an entirely different group of biomimetic adhesives. An adhesive based on the complex coacervate tubeworm adhesive theory was formed through the combination of synthetic polymers with phosphate, amine and catechol side-groups, in a similar molar ratio to that seen in tubeworm cement (Shao et al., 2009). Biocompatibility of the adhesive was tested in the rat model, where skull fractures were repaired with the complex coacervate adhesive. The adhesive was not cytotoxic, bonded with the bone, did not mix with blood or affect the underlying brain tissue, began to degrade after two weeks and did not interfere with normal bone healing (Winslow et al., 2010).

Bioadhesives have not only inspired glue mimics but have also led to innovative design of adhesive films for bandages. Chitosan has been produced as films that are soft and flexible with adhesive strength and biocompatibility comparable to currently used, synthetic wound dressings (Khan et al., 2000). The nanoscale features of films have also been influenced by the natural world, with gecko-inspired tissue adhesives that have nanoscale pillars to mimic the spatula of gecko foot setae. ‘Geckel’ is an adhesive tape that was produced with inspiration from both the gecko and the mussel (Lee et al., 2007). While the nanopillar structure of the tape resulted in adhesion in dry conditions,

the presence of water proved to be an obstacle. To get around this the nanopillar tape was coated in a synthetic polymer featuring DOPA. Another such adhesive film was created with a biocompatible and biodegradable elastomer, then coated with a thin layer of oxidised dextran (which has aldehyde function groups that act as a tissue adherent); the resulting tape caused a minimal inflammatory response in live rats and in the future such adhesives could be used to replace stiches and staples, as sealants and as grafts (Mahdavi et al., 2008).

1.4 Aims

The wider community that is dedicated to barnacle bioadhesion research shares the aim of searching for novelties in underwater adhesion, which will hopefully lead to biotechnological advances. As can be seen by the many unanswered questions surrounding the process of adhesion in barnacles, there is a vast amount of room for further studies of the various aspects of this phenomenon. The current study focussed on the stalked barnacle *Lepas anatifera* in order to address the bias towards acorn barnacles that exists in barnacle adhesion literature. Emphasis on species-specific comparisons was one of the objectives of this study; the conservation of functionally important domains across many barnacle species may provide researchers with a key to understanding the mechanisms at work in barnacle adhesive. In order to systematically characterise the adhesive of *L. anatifera* our study was divided into several parts, each with specific aims:

1. To establish protocols for collecting and cultivating *L. anatifera* adults and larvae.

2. To use histology, electron microscopy and histochemistry to examine the structure and chemistry of the adhesive apparatus (the adhesive gland and secretory canals) as well as the secretion itself as it appears within the secretory canals. Within this overlying purpose we aimed to provide a true account of these structures, as previous accounts were of acorn barnacle species and disagreed somewhat. Also, to compare this adhesive system with other biological adhesive systems and to use the structural details to form a picture of the secretory process.

3. To investigate the chemistry of the cured adhesive using Raman spectroscopy, FTIR spectroscopy and energy dispersive spectrometry techniques (SEM-EDS and TEM-EDS), and to compare the results from *L. anatifera* with recent studies of acorn barnacle adhesive. Also, we aimed to provide the first account of the elemental features of the adhesive glands of *L. anatifera*.

4. To elucidate the number of proteins within the adhesive through SDS-PAGE, search for homologous proteins and if only novel proteins are found, elucidate primary structure information using mass spectrometry and molecular techniques.

5. To use antibodies raised against acorn barnacle adhesive proteins to investigate the localisation of some barnacle adhesive proteins (cp-52k, cp-68k and cp-100k) and to search for the *L. anatifera* homologues of barnacle adhesive proteins (cp-19k, cp-20k and cp-100k), using published and unpublished protein and cDNA sequences from acorn barnacles and the stalked barnacle *P. pollicipes* (EST sequences) to design degenerate primers.

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2 The collection and long term cultivation of the adult goose barnacle *Lepas anatifera*

2.1 Introduction

Barnacles are sessile, filter feeding crustaceans and are of huge interest to scientists due to the strong adhesive that they produce. This adhesive has allowed barnacles to become notorious biofoulers of man-made marine structures, a costly problem that has led to many investigations of barnacle adhesive. Adding further fuel to the desire to understand this natural phenomenon is a need for underwater adhesives that can be applied and cured in wet environments, a challenge that nature has overcome in several different ways but humans have not. Almost all recent publications in the area of barnacle adhesive use acorn barnacles as research models, which is likely a reflection of availability. Stalked barnacles are often pelagic, such as the neustonic goose barnacle *Lepas anatifera*, a large staked barnacle that opportunistically adheres to any available floating substrate. Floating populations of *L. anatifera* are subject to the movements of wind and water currents, making their location unpredictable. Yet in theory, adherence to floating substrates is a suitable factor for captive cultivation; stalked barnacles do not reattach after removal from their substrate and do not thrive when kept free from their substrate (Patel, 1959), however a floating substrate can easily be moved into a lab environment.

2.1.1 Distribution and life cycle of *Lepas anatifera*

L. anatifera has a wide distribution; while generally considered to be a tropical-subtropical species it is found across the North Atlantic and in the North Sea (Bainbridge & Roskell, 1966; Sneli, 1983). The presence of this species in the waters around Ireland is indicated by reports of wide-scale strandings on beaches on the south and west coasts, after strong westerly or north-westerly winds (Cotton et al., 2006; Minchin, 1996). *Lepas anatifera*, like many other stalked barnacles, is a neustonic organism, existing at the ocean's surface. The floating lifestyle of these animals leaves it unclear whether their presence on Irish shores is a result of gregarious populations of adults floating to Ireland from elsewhere or an indication of breeding populations being present in Irish waters. Evidence from a survey of the North Atlantic and the Celtic Sea

suggests that breeding and early development do not occur there; only later stage nauplii larvae were observed and high numbers of larvae were only observed in years of above-average sea surface temperature (Bainbridge & Roskell, 1966). The same survey suggested that breeding in wild populations most likely takes place during summer months, with larval development occurring over several months. Their life cycle in the wild has not been observed; it can be assumed to be similar to that of other barnacles, however the timing of development, food requirements of each stage and temperature requirements are unknown. One previous attempt to cultivate the larvae of *L. anatifera* found that it took 57 days for the larvae to reach cyprid stage after hatching, which is a far longer development time than that of most other barnacle species (Moyse, 1963). However, in this study the laboratory-reared cyprids were smaller than those seen in the wild and did not survive to metamorphose into an adult barnacle (Moyse 1987). After settlement and metamorphosis it has been observed to take at least 30 days for *L. anatifera* to reach reproductive maturity in waters ranging in temperature from 24 to 26 °C. Growth was estimated to proceed at 0.5 mm daily until maturation was reached, after which point growth continued more slowly (Evans, 1958). In contrast to this, cyprid larvae of *L. anatifera* that were collected from the wild and settled in a laboratory setting at 25 °C achieved sexual maturity and released nauplii within 13 days (Green et al., 1994).

2.1.2 Cultivating stalked barnacles

Few studies have attempted to cultivate stalked barnacle species in a laboratory environment. A study by Patel (1959) described maintaining *L. anatifera* that had been removed from a buoy. The barnacles were kept in an aquarium in small dishes for approximately one month, unattached to a substrate. This study found that ovarian development occurred between 15 and 25 °C but fertilisation occurred only above 19 °C, while breeding was not possible above 30 °C. Self-fertilisation did not occur. At 25 °C it took 7 days for nauplii to hatch after fertilisation. Conclusions drawn suggested that it is possible that breeding may occur over a larger temperature range in the wild, where the species' natural food source is available, and that *L. anatifera* is thought to be less able to survive starvation than sessile barnacles (Patel, 1959). A more recent attempt to cultivate stalked barnacles utilised *Lepas anserifera* (Inatsuchi et al., 2010): adult barnacles were collected and kept in laboratory aquaria for 50 days in unfiltered

running seawater. The barnacles were fed *Artermia salina*, which were hatched directly in the aquaria twice a week. Three temperature treatments were used (19, 24 and 29 °C) and it was found that temperature had an effect on the maturation to reproduction, with higher temperatures producing higher growth and a higher number of reproductive animals. However, no animals produced eggs during the cultivation period and many animals died in all treatments (Inatsuchi et al., 2010).

In contrast to this, some acorn barnacle species are considered relatively easy to cultivate in the lab, as exemplified by El-Komi and Kajihara (1991). The acorn barnacle species *Amphibalanus amphitrite, A. eburneus* and *Balanus trigonus* were cultured for 2 ½ years, kept at 20 °C and fed *Artemis sp.* and phytoplankton culture. Breeding continued throughout this entire period, with multiple broods of embryos being produced each month. Breeding ceased when individuals were kept at 10 °C and ceased when individuals were cultivated without food. On return to normal conditions all animals resumed breeding.

There have been several past attempts to cultivate larvae of stalked barnacles, however a successful method of rearing stalked barnacles from their larval form has never been found. The development of successful methods to cultivate stalked barnacles from larvae could have applications for the aquaculture of commercially valuable species such as *Pollicipes pollicipes*, which are highly prized as an edible delicacy in Spain and Portugal and to that end natural populations are overexploited (López et al., 2010). Edible giant species of acorn barnacle such as *Austromegabalanus psittacus* are already being considered as feasible options for large scale commercial aquaculture, however their complete life-cycle has also not been reproduced in captivity, with cultivation proceeding using wild larval stock (López et al., 2012).

An account by Moyse (1963) described the rearing of several species of barnacle nauplii, including *L. anatifera*, and provided a comparison of different food sources for best survival. The most important factor in larval survival was considered to be a relationship between the geographical range of each species and the suitability of different types of algae as food. It was also suggested that different species may require different growth factors, thus leading to a preference for different sorts of algae. A later study by Stone (1989) reiterated that the morphology of the filtering apparatus of

barnacle nauplii reflects the food availability at their geographical range and also linked the diet with the timing of larval release, with continuous breeders able to survive on diatoms and flagellates, while spring breeders fared well on diatoms and summer breeders developed on flagellates. Another study of stalked barnacles by Lewis (1975) focussed on *Pollicipes polymerus*. Larvae were successfully reared and all larval stages described, however few cyprids settled; those that did settle only did so in close proximity to adult barnacles. The study does not mention whether the settled cyprids successfully metamorphosed.

2.1.3 Aims

The primary aim of this study was to collect live specimens of *L. anatifera* and maintain a captive population for an extended period of time in a laboratory environment, in order to have samples available for further studies whenever needed. Furthermore, we aimed to discover a method of cultivating larvae through the nauplii and cyprid stages and achieving successful metamorphosis in a laboratory environment, thus ensuring a source of samples in perpetuity. As adhesion to the substratum first occurs during settlement of the cyprid larva, this process could have been further explored had access to a substantial number of larvae been possible.

2.2 Methods and results: sampling

2.2.1 Onshore sampling

Samples of *Lepas anatifera* were searched for primarily throughout the summer of 2010, through the use of a beach-combing method. The stranding of stalked barnacles is unpredictable but a previous report indicated that best results would be following westerly winds, recent off-shore storms and high tides (Minchin, 1996). The sporadic nature of these events resulted in beach-combing being a time consuming method of sampling and in order to maximise results and conduct a more directed search, assistance from members of the public was increasingly relied on. Direct approaches were made to groups such as fishermen and surfing clubs, while a more general appeal was made via a press release that was featured in many national and regional newspapers and radio shows. Figure 2.1 shows the various locations where specimens of *L. anatifera* were located during the course of this study, by both the author and various colleagues.

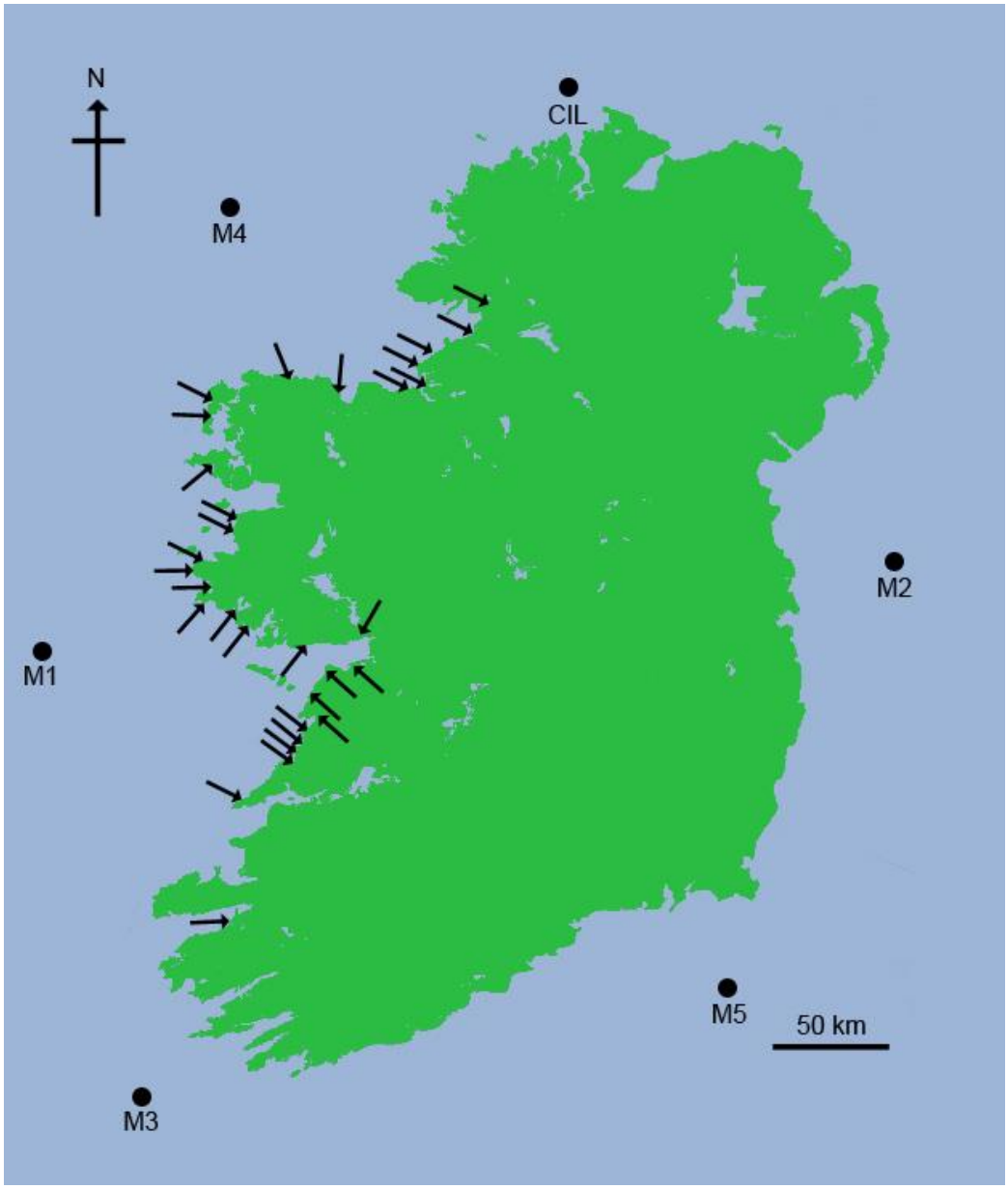

Figure 2.1: Map of Ireland with onshore sampling locations marked by arrows and data buoys marked by filled circles. M-marine institute data buoys, CIL-Commissioners of the Irish Lights buoy.

Evidence from various sources indicated that the most likely time to find *L. anatifera* on the Irish coastline would be late summer to early autumn, thus sampling efforts were focussed on the months of June, July, August, September and October. *L. anatifera* was found in the largest numbers in August and September.

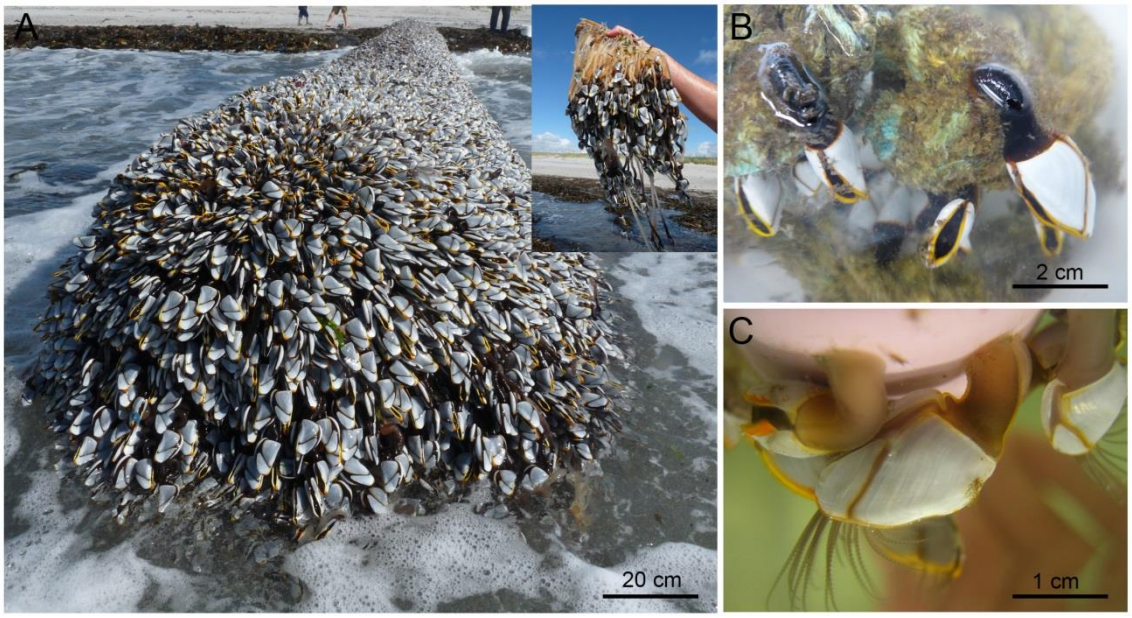

Figure 2.2: Results of onshore sampling. A) large wooden pole washed ashore in County Clare 2010 (inset: section of wood that was removed for transport); B) rope with *L. anatifera* attached, found southwest of Ireland 2012. C) plastic container with *L. anatifera* attached, County Galway 2010.

Samples of live *L. anatifera* (Figure 2.2) were immediately placed into buckets of fresh seawater (taken from the ocean), with lids placed on top. Experience showed that a large group of *L. anatifera* could quickly use up the available oxygen, thus while in transport oxygen tablets were added to the water. Upon arrival the samples were maintained in their transport containers, placed in a controlled temperature room (18 °C) and air pumps were used to supply oxygen to the water. Samples were left overnight to acclimatise to the room, and then placed in a recirculation aquarium.

2.2.2 Offshore sampling

The maintenance of buoys situated in the ocean around Ireland gave further opportunity for the collection of *L. anatifera*. Data buoys belonging to the Marine Institute (MI) and the Commissioners of the Irish Lights (CIL) were examined (Figure 2.1). Buoys belonging to the MI were towed to shore by the Celtic Voyager and lifted out of the water onto a pier, while the CIL did not tow their buoy through the water but lifted it onto their ship. All the buoys examined over the course of this study were dominated by a single fouling species, either mussels or seaweed, with small numbers of other species, such as barnacles, being present. Small numbers of *L. anatifera* were found on the CIL buoy (2009) and the Marine Institute M3 (2010), M4 (2009) and M5 (2012) buoys (Figure 2.3).

Figure 2.3: Results of offshore sampling: A) M3 buoy being lifted out of the water; B) *L. anatifera* attached to the M3 buoy by a large adhesive plaque; C) an immature egg lamellae being extruded by an adult *L. anatifera*.

Samples of *L. anatifera* attached to buoys were generally large, with wide adhesive plaques. The samples were carefully removed by inserting a flat razor blade between the adhesive plaque and the substrate, taking care to not damage the body of the animal. Only healthy, undamaged samples were collected. Animals were transported in the same manner as described above.

2.3 Methods and results: cultivation

2.3.1 Culture conditions

Samples of *L. anatifera* were maintained for over a year (approximately 14 months, at which point remaining samples were sacrificed and fixed for future work) at 18 °C in a 300 L aquarium fitted with a recirculation system to constantly filter and clean the water (Figure 2.4). As water left the aquarium it moved through a filter mat into a bacterial bed and a chamber of activated carbon, to remove unwanted chemicals. After filtration, water passed through a protein skimmer (Schuran) to remove excess dissolved protein before being pumped back into the aquarium. However, large numbers of barnacles often produced too much waste to be removed by the filter system alone, thus once a month ¼ of the water was removed from the aquarium and replaced with fresh seawater. Healthy barnacles that were maintained in this manner thrived for several months, with some remaining in the aquarium for over a year. The animals continued to reproduce and produce viable larvae for most of this period.

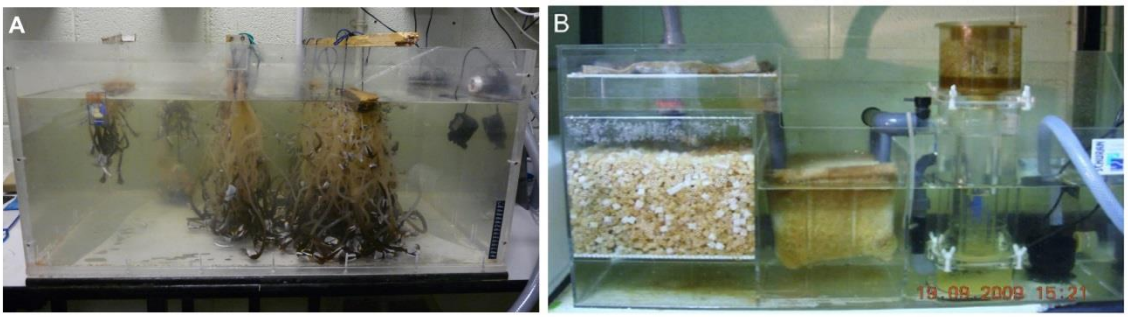

Figure 2.4: A) recirculation tank used for cultivation of adult *L. anatifera*; B) filter system for recirculation tank.

The brine shrimp *Artemia salina* (Zebrafish Management Systems) was cultured as a food source for *L. anatifera*. *A. salina* cysts were hatched in enriched seawater, which was a mixture of seawater and live plankton (*Nannochloropsis spp., Isochrysis galbana, Rhinomonas spp.* and *Phaeodactulym triconutum*, supplied by NUI Galway Zoology Carna Research Station) in 2 L culture flasks (Zebrafish Management Systems). *A. salina* was then cultured for three days at 25 °C and further enriched on the third day with 5 mL each of the invertebrate foods S.parkle and S.espresso (Inve Technologies). The three day old *A. salina* were separated from their culture media by phototaxis and one flask was fed to *L. anatifera* each day. For a short time this diet was supplemented

by a slurry of fish and mussel but this had no noticeable benefit and fouled the water, thus was discontinued.

The population of barnacles being cultured decreased the most during the winter, when a technical fault resulted in the temperature of the culture room and aquarium dropping to 11 °C. Specimens that survived through the winter no longer reproduced. Other factors adversely affecting the survival of cultured barnacles included fungal infection and polyp infestation. To mitigate fungal and possible bacterial infections all new samples were treated with fungizone (1% v/v) (Thermo Scientific) and penicillin-streptomycin (0.2% v/v) (Thermo Scientific) before being transferred into the large recirculation aquarium. Hydrozoan polyp infestation of the aquarium, recirculation apparatus and the barnacles themselves appeared after several months of cultivation and to keep it to as low a level as possible the aquarium and filters were cleaned regularly and heavily infected individual barnacles were removed from the aquarium. Animals that survived in captivity for a year or more showed signs of decalcification; UV treatment and calcium supplements were used to combat this issue but no improvement was seen (Figure 2.5).

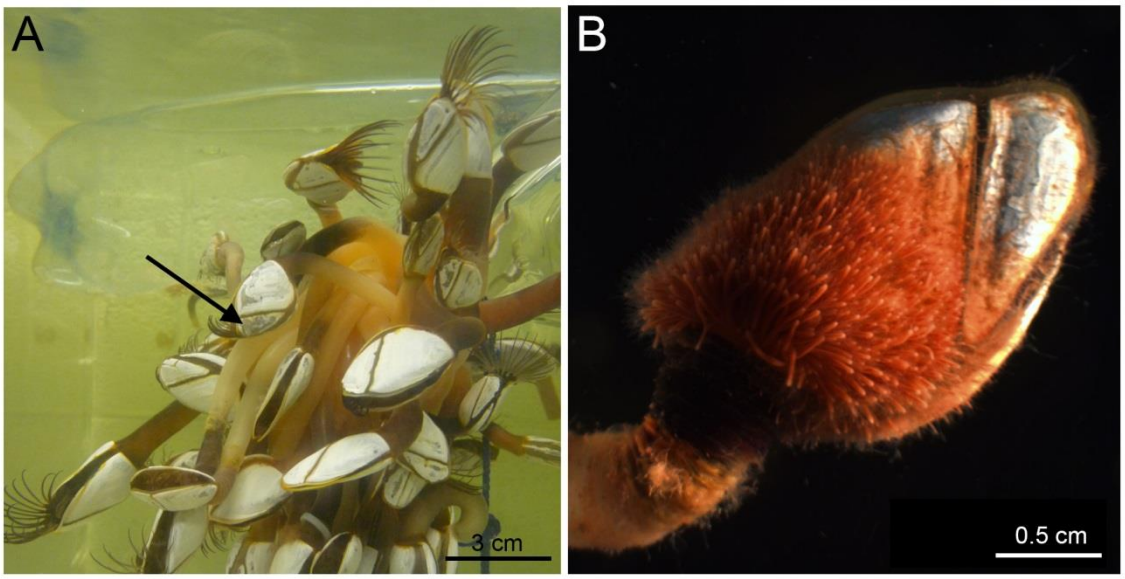

Figure 2.5: Adverse effects of captivity: A) decalcification of the capitular plates (arrow); B) polyp infestation.

Over several months the moult cycle of select individuals was tracked by placing them in individual units within the aquarium. Water, oxygen and food could flow through these units but cirral moults could not. Units were checked twice daily for cirral moults

and in this way the moulting cycle for these individuals was recorded. The length of each cycle was quite variable, ranging from 6 to 46 days (mean 20.8, standard error 1.98), with variation not only seen between individuals but also between moult cycles for each animal.

2.3.2 Cultivation of larvae

Reproductive adult barnacles released egg lamellae, which were removed from the recirculation aquarium into a smaller, aerated vessel for hatching. Egg lamellae were also collected from barnacles that were washed ashore or attached to buoys but could not be removed from their substrate intact. Immature egg lamellae were bright blue, with a rubbery appearance, while mature egg lamellae were a pinkish-white colour. Mature egg lamellae hatched within an hour of being released by the animal, while immature lamellae took up to 3 days to change from blue to pink, followed by larval release. The larvae hatched from the egg lamellae as stage-I nauplii and quickly metamorphosed into stage-II nauplii (within approximately 15 minutes) (Figure 2.6).

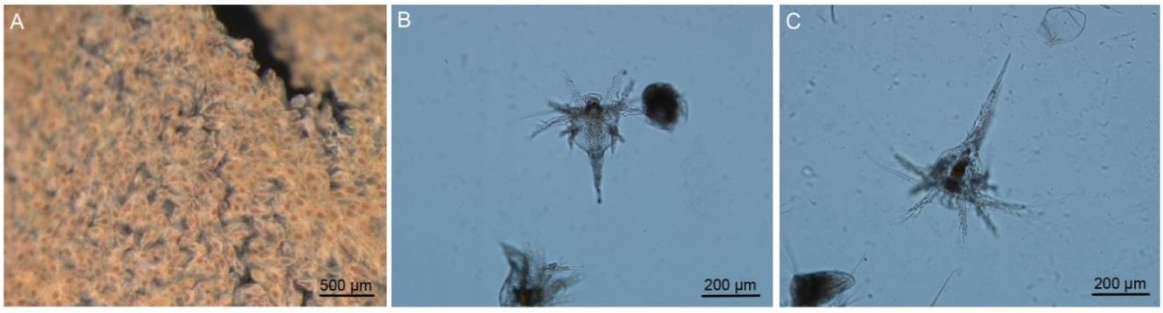

Figure 2.6: A) lamellar mass of embryos ready to hatch, image by Paul McEvilly; B) stage I nauplii; C) stage II nauplii.

Various containment vessels were used in attempts to cultivate *L. anatifera* nauplii, including aquaria (5 L to 50 L), conical flasks, beakers and bottles (Figure 2.7). The most severe impediment to the survival of the nauplii appeared to be their inability to remain suspended in the water column. Various efforts were made to keep the larvae afloat, including the use of a small kreisel tank, placing vessels on rocking tables, agitating the water with magnetic stirrers and placing aeration tubes at the base of the vessel. Overcrowding was also detrimental to the survival of the nauplii, thus efforts were made to place no more than 20 nauplii in each litre of water. The temperatures that nauplii cultivation vessels were kept at ranged from 18 to 25 °C. Various feeding regimens were also used, including a mixture of plankton (*Rhinomonas spp.*,

Nannocloropsis spp. and *I. galbana*) and pre-made invertebrate nutrients (S.parkle and S.presso (Kinetica) and Preis-Microplan (Preis-aquaristik)).

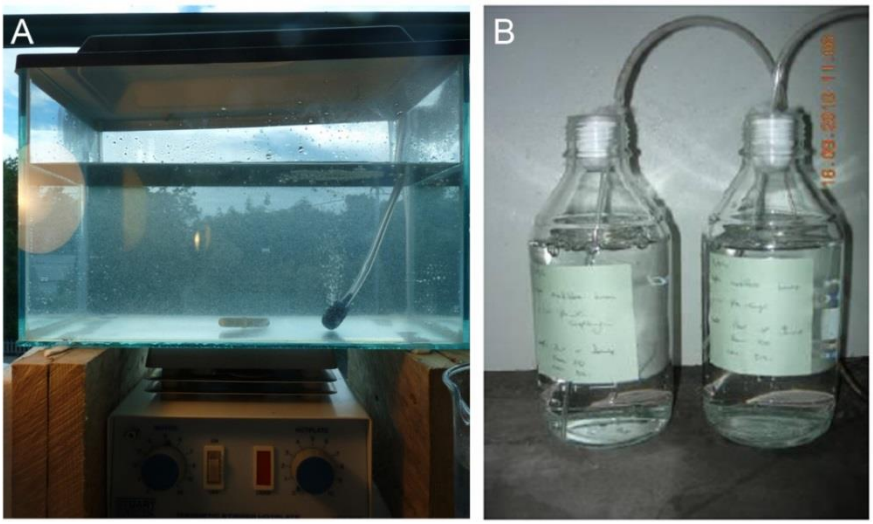

Figure 2.7: Embryo cultivation arrangements: A) 15 L aquarium with magnetic stirrer and gentle oxygen flow at base of tank; B) 1 L bottles with very gentle oxygen flow at base of bottle, image by Paul McEvilly.

Cultivation of nauplii was largely unsuccessful, with a large amount of mortality occurring at stage-II or -III. Stage-III was reached about 7 days after hatching, stage-IV at 16 days and stage-V at 25 days. A single stage-V nauplii was observed in the course of this study (Figure 2.8). This was considered to be the most successful cultivation attempt and was raised in a 1 L bottle at 25 °C with an aeration tube placed at the base of the bottle, releasing a very slow stream of bubbles (approximately 40 per minute) to gently agitate the water. The nauplii of this vessel were fed exclusively on preis-microplan invertebrate food. Instructions for this product suggest 1 drop of the mixture per 15 L of water, however this was modified by a factor of 10 due to necessity: 1 drop of preis-microplan was mixed with 15 mL of seawater in a falcon tube, then a drop of the resulting mixture was added to the cultivation vessel once per week.

Figure 2.8: A) stage III nauplius; B) stage IV nauplius; C) stage V nauplius. Images by Paul McEvilly.

2.4 Discussion

Over the course of this study a successful method of locating *L. anatifera* was devised. *L. anatifera* were found with the most regularity in the months of August, September and October. The most animals were found in County Clare, however it is hard to say whether this is a true reflection of *L. anatifera* strandings or a result of effort being most expended in County Clare, on account of ease of access. Our method of searching was largely based on anecdotal evidence and personal communications from various beach observers and fishermen, who informed us that stalked barnacle species were known to be stranded on the west, particularly the southwest, coasts during strong northwest winds and strong tides in late summer and autumn. Recently Cotton et al. (2006) described mass stranding of the stalked barnacle *Dosima fascicularis* in Counties Mayo and Sligo, an occurrence never before seen in this area, while an older publication discussed the presence of thousands of colonies of *D. fascicularis* and small numbers of *L. pectinata*, *L. anserifera* and *L. anatifera* on various substrates on the Irish coastline (Minchin, 1996). The conclusions drawn by the authors validate our own bias towards sampling on the south-west coasts for the duration of this study. In addition to our own efforts to find *L. anatifera*, appealing to the public for help in locating samples ultimately provided only a moderate result. While it may be possible to place substrates in the ocean to be colonised by barnacles such as *L. anatifera*, such a method may be not be very successful. It was clear when examining data buoys that barnacles were never the dominant fouling species, though their presence on stationary objects such as buoys indicated that at the final larval stages are present in Irish waters. Utilising barnacle settlement inducing protein may be an option for more direct collection of barnacles in future studies. An advantage of this would be the possible estimation of settled barnacle age.

The results of this study showed that healthy specimens of *Lepas anatifera* could be successfully maintained in captivity for up to 14 months or more, during which time they were able to continue to produce adhesive. The normal lifespan of this species is unknown; its pelagic lifestyle is not conducive to observational studies, plus the age of the barnacles was unknown at collection. While it was observed that the barnacles grew rapidly from miniscule, recently metamorphosed individuals to adult size, their longevity as adults is unknown. Therefore we cannot say whether the life span of the

captive barnacles differed from that of wild barnacles. It is likely that the captive barnacles were maintained at a lower level of health than wild barnacles, as their food source was limited to a single species and they were subjected to various detrimental factors such as decalcification and polyp infestation. The short duration of cultivation of *L. anatifera* in the past is likely to be due to the aims of the individual studies (Inatsuchi et al., 2010; Patel, 1959), as opposed to the difficulty of cultivation. However, while the results of the current study show that it is possible to maintain adult *L. anatifera* for a very extended period, after extensive trials we were unable to successfully cultivate the larvae of *L. anatifera* successfully to cyprid stage and adulthood.

Cultivation of larvae to cyprid, settlement and metamorphosis would facilitate a huge range of further investigations of stalked barnacle adhesive and comparisons with acorn barnacle adhesive. Settlement onto specific substrates such as germanium wafers would allow for *in situ* ATR-FTIR experiments, as was performed with adult acorn barnacles by Barlow et al. (2009); this method could then be extended to show the change in chemical profile as the cyprid adhesive is replaced by adult adhesive. Settlement on transparent substrates could be used for microscopy studies of intact adhesive plaques, while settlement onto substrates of various compositions could show whether the adhesive interface that *L. anatifera* produces takes on different morphologies depending on the substrate, similar to investigations of acorn barnacle adhesive (Raman et al., 2013; Sullan et al., 2009; Wiegemann & Waterman, 2003). Also, raising adult barnacles from cyprids in a controlled environment would give researchers more confidence that the adhesive used for subsequent experiments was not contaminated by external environmental factors. While the results of this study and previous studies show that cultivating large stalked barnacle larvae is a sensitive and difficult task, they do not indicate that the task is impossible, only that stalked barnacle larvae cannot be cultivated on a small scale.

The relative ease of cultivating acorn barnacles from larvae, compared to stalked barnacles, has led to a huge bias in our understanding of barnacle biology, from the factors required for larvae to thrive to the shared mechanisms of adhesion utilised by all barnacles. Successful cultivation of stalked barnacle species would not only allow for more thorough investigations of many aspects of barnacle biology, but could have a huge commercial benefit.

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3 Unusual adhesive production system in the barnacle *Lepas anatifera*

Published as: Jonker, J.-L., von Byern, J., Flammang, P., Klepal, W., & Power, A. M. (2012). Unusual adhesive production system in the barnacle Lepas anatifera: An ultrastructural and histochemical investigation. *Journal of Morphology*, *273*(12), 1377-1391.

3.1 Introduction

Marine organisms such as barnacles, mussels and tubeworms create natural adhesive substances and are of great interest in the search for biomimetic adhesives with applications in wet environments. The most widely studied adhesive glandular systems are those of the acorn barnacle *Megabalanus rosa* (Arthropoda), the mussel *Mytilus edulis* (Mollusca) and the tubeworm *Phragmatopoma californica* (Annelida) (e.g., Kamino, 2010a; Waite, 2002; Wang et al., 2010). These organisms use proteinaceous secretions to achieve permanent attachment (Stewart et al., 2004; Waite, 1987; Walker, 1970). Barnacle adhesive is secreted as a liquid of very low viscosity that solidifies to form a rubbery mass and then hardens. The mechanism by which the barnacle adhesive transforms from liquid into strong, hard, cured adhesive is unknown but is thought to be distinct from the adhesive processes employed by mussels and tubeworms. The adhesive abilities of the mussel and tubeworm both rely on post-translational modifications (PTMs) of the adhesive proteins. The hydroxylation of tyrosine into L-3,4-dihydroxyphenylalanine (DOPA) and the phosphorylation of serine (pSer) confer adhesive and cohesive ability through cross-linking and metal-binding (Zhao et al., 2005; Zhao & Waite, 2006). There has been no indication that these modifications are present in the four barnacle adhesive proteins (cp-19k, cp-20k, cp-52k and cp-100k) fully characterised and published so far (Kamino et al., 2000; Kamino et al., 2012; Mori et al., 2007; Urushida et al., 2007); however, at least six further proteins are thought to exist (Kamino, 2006; Naldrett & Kaplan, 1997). Two of the fully characterised barnacle proteins have adhesive properties on specific substrates (Mori et al., 2007; Urushida et al., 2007) and it has been suggested that the largest protein may form amyloid-like cross-β-sheet structures (Kamino et al., 2000), but no evidence has been found to suggest covalent cross-linking of proteins in the barnacle adhesive (Kamino, 2010a). Although a recent theory has proposed that cross-linking mediated by transglutaminase from haemocytes is an important molecular adhesive mechanism in barnacles

(Dickinson et al., 2009), this hypothesis was based on indirect evidence (Kamino, 2010b) and requires further verification.

In addition to potential molecular novelties, there are also morphological differences between barnacles and other adhesive producers. The glandular tissues that produce marine adhesives generally consist of more than one type of secretory cell, each producing distinct substances, but this is apparently not true for barnacles. For example, the mussel produces at least five novel proteins for the construction of the byssus (Waite, 2002), the secretion of which is brought about from three glands within the foot of the mussel, which add components in a stepwise manner (Tamarin, 1975; Tamarin & Keller, 1972; Walker, 1987). Three unique proteins have been sequenced from the tubeworm (Zhao et al., 2005), although many more unknown proteins are suspected to form a part of the complex (Endrizzi & Stewart, 2009). The adhesive glands of tubeworms consist of multiple secretory cell types and morphologically distinct secretory granules, which remain physically separated until extruded from the body (Wang & Stewart, 2012)). In contrast to these systems, the ten or more proteins found in barnacle adhesive are all apparently produced by a single cell type (Lacombe & Liguori, 1969; Walker, 1970). This raises questions about regulation of protein secretion, the activation of the adhesive hardening process and the prevention of premature hardening of adhesive while still within the tissues of the barnacle.

Several previous studies exist on the morphology of adhesive glands in barnacles, including two brief ultrastructural accounts (Koehler, 1889; Lacombe, 1970; Lacombe & Liguori, 1969; Walker, 1970, 1978). In this paper, we aimed to describe the finer structure of this ‘unicellular’ adhesive gland system, specifically the process by which the glandular secretion passes from the point of synthesis in the cell to the outside of the animal. We also examined the histochemistry of the gland in light of current theories of marine adhesion, such as the involvement of amyloid and of the PTMs DOPA and pSer. Oxidisation of DOPA into quinone and semi-quinone forms has cohesive functionality in mussels (Wilker, 2011) and quinone cross-linking has also been hypothesised in the past for barnacles (Lindner & Dooley, 1972; Shimony & Nigrelli, 1971). The identification of phenols and polyphenoloxidase in the adhesive glands of barnacle larvae (Walker, 1971) raised the possibility of such a mechanism being at work, but the larval and adult adhesives are likely to be distinct (Aldred & Clare, 2008; Power et al.,

2010; Walker, 1971). However, a single study has reported phenolase activity in adult gland cells, which indicates similarity with the larval system (Lindner & Dooley, 1972). Other studies have shown tyrosine to be present in adult adhesive (Naldrett, 1993; Walker, 1972) and gland cells (Fyhn & Costlow, 1976; Karande & Gaonkar, 1977; Walker, 1970), raising the possibility of DOPA involvement in adult adhesion. Although neither DOPA nor pSers have been seen in the four proteins characterised to date, their presence has not yet been ruled out in the uncharacterised proteins. We aim to investigate the presence of these PTMs in the glandular tissues of barnacles using specific assays.

In addition to evidence for PTMs, we searched for evidence of transglutaminase-producing haemocytes within the adhesive 'apparatus' (the gland cells and their associated drainage network) in order to investigate the hypothesis of Dickinson et al. (2009). Our analyses were conducted on *Lepas anatifera*, a stalked barnacle with a pelagic lifestyle. This species grows to a much larger size than acorn barnacles and adheres to floating debris in the open ocean, including diverse surfaces such as wood, plastic, glass, skin and feathers. By comparison with acorn barnacles in the order Sessilia very little research has been conducted using stalked species (orders Lepadiformes and Scalpelliformes); to date there has been only one histological study of the adhesive secretory tissues of stalked barnacles (Lacombe & Liguori, 1969) and two biochemical studies of the adhesive of stalked barnacles (Barnes & Blackstock, 1976; Walker & Youngson, 1975). Finally, as it has been proposed in the past that glandular secretion of barnacle adhesive coincides with the moulting cycle (Fyhn & Costlow, 1976), histochemical analyses were applied to animals at different stages of the moulting cycle.

3.2 Materials and methods

Samples of *Lepas anatifera* were collected opportunistically around the Irish coast throughout 2010 and 2011 from debris washed up on beaches or from data buoy cleaning missions. The large size and distinct morphology of stalked barnacles (including a thick adhesive plaque at the base of the animal) allowed them to be easily removed from a substrate without causing damage to the secretory structures within the stalk. Samples were either fixed immediately upon collection or maintained at constant temperature (19 °C) in an aquarium with a recirculating current and biological filtration of waste material. All samples consisted of mature animals with a capitulum length ranging from 5 mm to 5 cm. Captive animals were fed on 3 day old *Artemia salina* enriched with a combination of microalgae: *Rhinomonas spp*., *Nannochloropsis spp*. and *Isochrysis galbana*. It was possible to maintain adult barnacles in this manner for more than 12 months.

Investigations into the gross morphology and chemistry of the adhesive system included a comparison of the glandular structures at different moult stages. For this some animals were maintained in individual units within the aquarium so that the moulting cycle could be tracked. Units were checked twice daily for cirral moults. The intermoult period was variable and ranged from one week to, more occasionally, one month in length. For the majority of individuals, the moulting cycle was represented by fixing animals for histology on the day of moulting, five days after moulting and ten days after moulting.

For histological and histochemical analyses samples were dissected into pieces measuring 1 cm^3 or less and fixed in Carnoy's or AAF fixatives (Kiernan, 2008) for 3 hours, with continuous agitation. Samples were dehydrated in graded alcohol and embedded in paraffin wax and sections were cut at a thickness of 5-7 µm (Leica biocut 235). A total of 25 individual barnacles were examined, some of known moult-stage and others unknown. The Heidenhain's AZAN stain (Kiernan, 2008) was used for general histology and a range of stains were applied for histochemical analysis (Table 3.1). For amyloid staining, Congo red and Thioflavin T were used and human kidney tissue with amyloidosis was used as a positive control (provided by University Hospital Galway). All light microscopy was performed using an Olympus BX51, with two polarising

filters inserted for viewing Congo red. Confocal microscopy (Olympus IX71 Fluoview 300) was conducted to determine Thioflavin T binding to tissues and autofluorescence was determined in unstained tissues (excitation 488 nm, emission 535-565 nm).

Table 3.1: Histochemical tests applied to the adhesive apparatus of *L. anatifera* embedded in paraffin sections.

Stain	For the localisation of	Control	Reference
Alcian blue (pH 1.0–2.5)	Acidic mucopolysaccharides	1.0 mol L^{-1} HCl (remove RNA)	(Pearse, 1960)
Azure A (pH 3.0–5.5)	Acidic proteins	1.0 mol L^{-1} HCl and deamination	(Kiernan, 2008)
Biebrich scarlet (pH 6.0–pH 8.0)	Acidic and basic proteins	Deamination	(Spicer & Lillie, 1961)
Congo red	Amyloid	Human tissue (amyloidosis)	(Highman, 1946)
Millon's reaction	Tyrosine containing proteins		(Baker, 1956)
Mercury Bromophenol blue (MBPB)	General proteins	Deamination	(Maiza et al., 1953)
Benzoylation + MBPB	Hydroxyl functional groups		(Kiernan, 2008)
Periodic acid Schiff's	1,2 Glycol carbohydrates	Acetylation	(Kiernan, 2008)
Thioflavin T	Amyloid	Human tissue (amyloidosis)	(Burns et al., 1967)
Thioglycollate ferric ferricyanide	Protein bound S–S and S–H		(Barnett & Seligman, 1958)

The presence of DOPA and pSer were investigated using chemical and immunohistochemical assays, respectively. Sections of the tubeworm *Sabellaria alveolata* were used as a positive control, as the adhesive gland tissues contain both DOPA and pSer. Five individuals of *L. anatifera*, of unknown moult-stage, were fixed in Bouin's fluid, embedded in paraffin wax and sectioned at 7 µm. For these experiments, sections were made of both the upper peduncle, containing the glandular apparatus, and the cured adhesive that was attached to the base of the peduncle. The Arnow assay (Arnow, 1937) was used to detect DOPA. After removing the paraffin and rehydrating, the sections were placed in 5% HCl for 5 minutes, 10% $NaNO_3$, 10% $Na_2MoO_4.2H_2O$ (in H_2O) for 5 minutes, followed by 1 mol l^{-1} NaOH for 5 minutes.

For the detection of pSer, sections were de-paraffinised, rehydrated and moved into PBS (pH 7.4). Non-specific binding was blocked by incubation for 30 minutes in PBS with 3% bovine serum albumin (BSA; Sigma-Aldrich), directly followed by incubation with monoclonal anti-phosphoserine antibodies (clone PSR-45, mouse ascites fluid; Sigma-Aldrich), diluted 1:100 in PBS-BSA, for 1 hour at room temperature. Sections were washed in PBS, then incubated for 30 minutes in anti-mouse immunoglobins conjugated to peroxidase (ImmPRE Reagent; Vector Laboratories) and washed again in PBS. Immunoreactivity was visualised using diaminobenzidine tetrahydrochloride

(DAB; Sigma-Aldrich) with Mayer's haemalum as a counterstain. Control reactions were performed by substituting the primary antibody with PBS-BSA and also by using the primary antibody mixed with pSer antibody inhibitor (Sigma-Aldrich).

For ultrastructural analyses, samples were sectioned into slices approximately 1 mm thick and fixed in modified Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 mol l^{-1} Na-cacodylate buffer, pH 7.2, with 10% sucrose) for 6 hours. Secondary fixation was performed using 1% osmium tetroxide (in 0.1 mol l^{-1} Na-cacodylate buffer, pH 7.2, with 10% sucrose). Samples were dehydrated in graded ethanol followed by propylene oxide, then infiltrated and embedded with Epon resin (Agar Scientific). Sections were cut at 1 µm (Leica Ultracut E) and stained with toluidine blue to indicate the general orientation of the section, after which ultra-thin sections were cut (80-100 nm) and stained with 1.5% uranyl acetate (30 minutes) and lead citrate (10 minutes) before analysis with TEM (Hitachi H7000). Samples examined in electron microscopy came from five different *L. anatifera* individuals, fixed on different occasions, all of unknown moult stage.

3.3 Results

3.3.1 Morphology of the glandular apparatus

The glandular ‘apparatus’ (the gland cells plus drainage network) of *L. anatifera* was analysed in mature animals; maturity was confirmed by the presence of extensive ovarian tissue. Only one type of secretory cell was associated with the glandular apparatus in the adhesive system. These cells were very large and were scattered amongst ovarian tissue in the peduncle (stalk), just below the capitulum (Figure 3.1A). The diameter of the cells ranged from 70 µm to 180 µm, with an irregular shape that varied from rounded to elongate. No evidence was seen for gland cells of different maturities, such as developing, mature and senescent cells, being present in *L. anatifera*. The adhesive secretion was released from the gland cytoplasm into a branched system of drainage canals that culminated in a pair of large principal canals, which delivered adhesive to the substratum (Figure 3.1B–E). The entire apparatus was surrounded by a continuous sheath of fibrous connective tissue, separating the adhesive system from other barnacle tissues, including the circulatory system. Within the connective tissue fibroblasts were observed: elongated cells with ovoidal nuclei and moderately dense cytoplasm. Inside the principal drainage canals, there was a thick layer of a cuticle-like substance lining the lumen, which further isolated the secretion contained within the adhesive drainage system (Figure 3.1E).

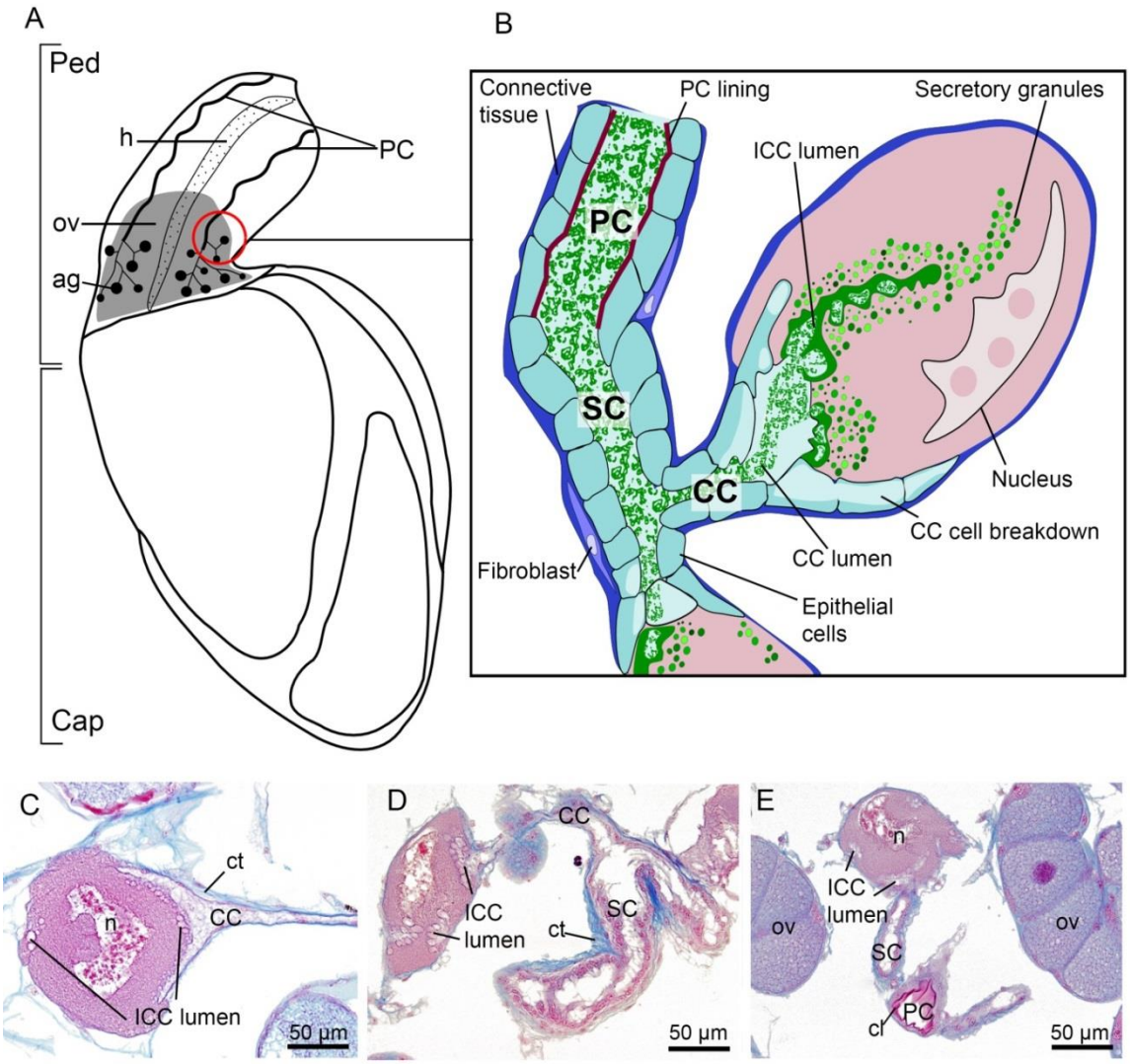

Figure 3.1: A) Diagram of stalked barnacle showing major structures within the peduncle. Adhesive glands are amongst the ovarian tissue. The gland drainage canals form a network that fuses into two principal canals, which extend up the peduncle, on either side of the haemolymph vessel. B) Diagram showing magnified view of an adhesive gland and the canal system. The nucleus of the cell is large, irregular shaped and contains nucleoli. The secretion (green) accumulates within the cytoplasm (pink) in granules of varying size and density, which either fuse to the ICC or release their contents into the adjacent cytoplasm. The lumen of the ICC is packed with both dense secretion and pockets of loose, flocculent secretion. The CC lumen forms through the cytoplasmic breakdown of the epithelial cells that form the canals, allowing the secretion to move from the ICC lumen into the CC lumen. The CC fuses with the SC, which in turn fuses with the larger PC, which has a thick cuticle lining. The entire apparatus is sheathed in a layer of connective tissue (blue), inside of which fibroblasts can be seen. C) Adhesive gland cell in which the vacuole-like lumen of the ICC can be seen adjacent to the CC. D) Collecting canal leading from the adhesive gland to the secondary canal. (E) Secondary canal extending between an adhesive gland cell and the principle canal. Ag-adhesive glands, cap-capitulum, CC-collecting canal, cl-cuticle lining, ct-connective tissue, h-haemolymph vessel, ICC-intracellular canal, n-nucleus, ov-ovarian tissue, PC-principal canal, ped-peduncle, SC-secondary canal.

Figure 3.1B schematically represents the main morphological features of the adhesive apparatus in barnacles. Within the adhesive gland cell, the adhesive secretion was synthesised and packaged into granules of varying size and density. The drainage of these granules from the gland cell began at the intracellular canal (ICC); a single, branched canal that formed inside each gland cell, apparently through the accumulation

and fusion of large secretory granules (probably the same structures that have previously been termed ‘vacuoles’ (Walker, 1970)). The details of the ICC could only be resolved with the use of electron microscopy (see below). It is noteworthy that, after secretion from the cell, the adhesive product was not divided into bound, separated structures of any sort. The adhesive secretion was transported to the substratum through a series of progressively larger, extracellular canals: the collecting canal (CC), secondary canal (SC) and principal canal (PC; Figure 3.1C-E). This was achieved via the ICC from each gland cell, which was in direct contact with a single CC. The latter consisted of epithelial cells with cytoplasm that was poor in organelles and which broke down to form a lumen. A number of CCs, from many gland cells, fused with the larger SCs, which in turn fused with one of the paired PCs. The PCs ultimately delivered the secretion to the outside environment. Principal canals were large, cuticle-lined structures meandering along the length of the peduncle, one on either side of the haemolymph space, until they reached the adhesive base of the animal (Figure 3.1A). The PCs were so large that, in some live individuals, they could be recognised with the naked eye.

The gland cells showed morphological evidence of high levels of protein synthesis. The large nucleus and presence of many nucleoli indicated that production of ribosomes was high (Figure 3.2A). The gland cell cytoplasm was very rich in rough endoplasmic reticulum (RER), typically in the form of swollen, rounded cisternae and less often as stacked cisternae (Figure 3.2B). The nucleus had a lobulated shape with many narrow folds which projected into the cytoplasm, greatly increasing the surface area of the nuclear membrane (Figure 3.2C-E). In some gland cells the cell membrane also had an increased surface area, with long, thin processes reaching over halfway through the cytoplasm, a feature that has also been reported by previous authors, such as Walker (1970). Many mitochondria were observed around the ICC and amongst the accumulated secretory granules.

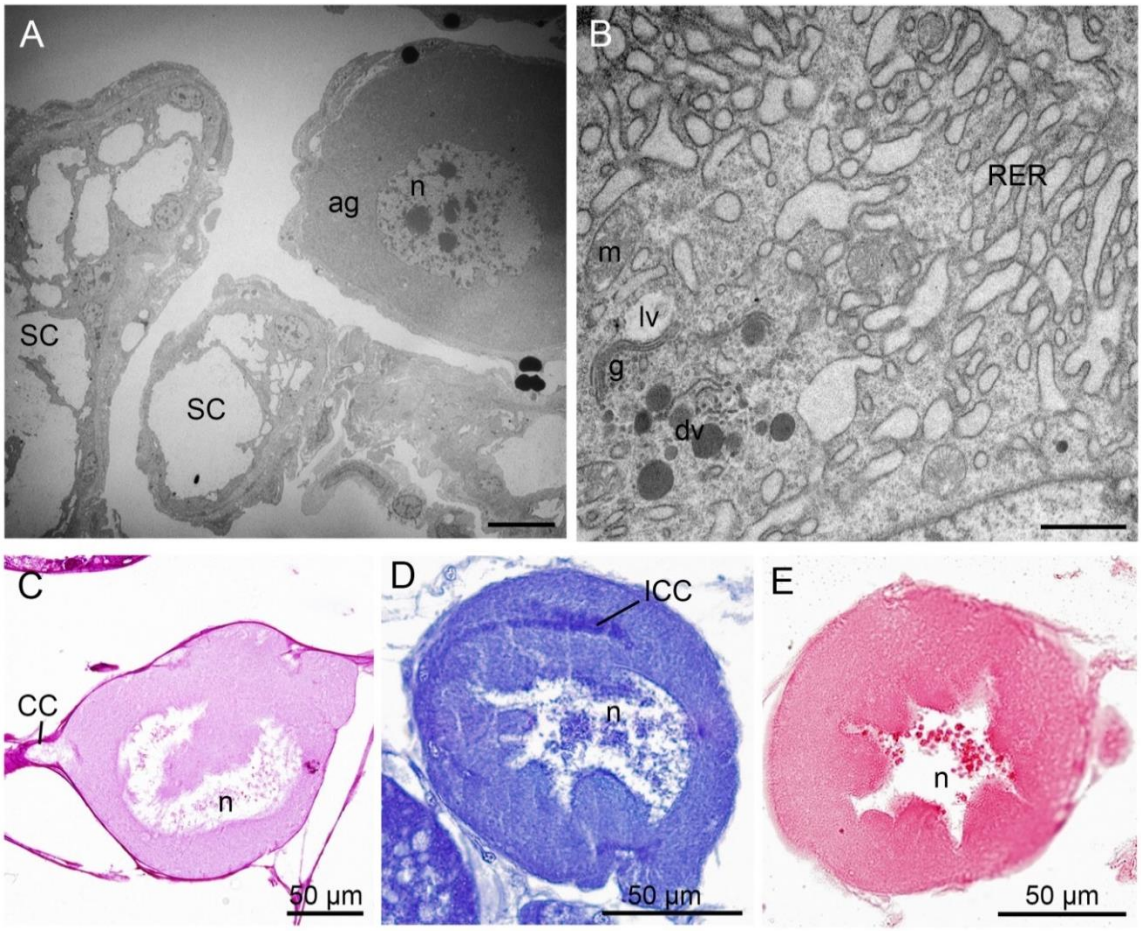

Figure 3.2: A) TEM micrograph of an adhesive gland cell; the large nucleus containing nucleoli can be seen and the gland is surrounded by portions of secondary canal. B) Cytoplasm with Golgi apparatus, with lucent and dense vesicles, and RER. C) Periodic acid Schiffs reaction showing a moderate amount of carbohydrate within the gland cell cytoplasm. D) Bromophenol blue reaction showing a high amount of protein within the gland cell cytoplasm, particularly in the ICC. E) Biebrich scarlet reaction at pH 6.0 showing the contents of the gland cell to include slightly acidic protein. Ag-adhesive gland cell, CC-collecting canal, ct-connective tissue, dv-dense vesicle, g-Golgi apparatus, ICC-intracellular canal, lv-lucent vesicle, m-mitochondria, n-nucleus, RER-rough endoplasmic reticulum, SC-secondary canal.

3.3.2 Chemistry of the gland cell

The gland cell cytoplasm was most reactive to stains for the detection of proteins (Table 3.2). Weak reaction by Azure A was seen at pH 3.5 and a stronger reaction by Biebrich scarlet at pH 6.0; however no reaction was seen by Biebrich scarlet at pH 8.0. Although a weak reaction was seen to Alcian blue at pH 2.5; this reaction disappeared when tissue was pre-treated with HCl to remove nucleic acids, thus reaction to Alcian blue was on account of a high concentration of RNA being present in the cytoplasm. The cytoplasm also displayed a moderate reaction for carbohydrates (Figure 3.2C-E; Table 3.2). Protein staining by Bromophenol blue was most intense in the area of the ICC, where the proteinaceous secretion was drained from the cell (Figure 3.2D). Benzoylation in combination with Bromophenol blue resulted in a mottled green-blue colouration within

the gland cell; this distinct reaction suggested that the proteins in the cell were rich in hydroxyl groups, indicating strong presence of the amino acids serine, threonine and/or tyrosine. The presence of tyrosine was confirmed by Millon's reaction. A strong positive reaction for monomeric cysteine (S–H groups) was observed only after the tissue was subjected to a reducing thioglycollate treatment, indicating that prior to this the untreated gland cell contained the dimer cysteine (S–S groups).

Table 3.2: Histochemical analysis of the adhesive gland cytoplasm of *L. anatifera.* Reactions defined as no reaction (-), weak reaction (+), moderate reaction (++) and strong reaction (+++).

Stain	Purpose	Reaction
Alcian blue pH 2.5	Nucleic acids and acid mucopolysaccharides	+
Alcian blue pH 2.5 after HCl pretreatment	Acid mucopolysaccharides	–
Azure A pH 3.0 after HCl pretreatment	Acid protein	–
Azure A pH 3.5 after HCl pretreatment	Acid protein	+
Biebrich scarlet pH 6.0 after HCl pretreatment	Acid protein	++
Biebrich scarlet pH 8.0	Basic protein	–
Congo red	Amyloid	–
Mercury bromophenol blue (MBPB)	General protein	++
Benzoylation + MBPB	Control/block hydroxyl groups	++
Millon's reaction	Tyrosine	+
Periodic acid Schiffs	Carbohydrate	++
Thioflavin T	Amyloid	+
Thioglycollate ferric ferracyanide	S–S groups	+

The adhesive gland cytoplasm stained negative for amyloid with Congo red (Table 3.2; Figure 3.3A, B). Although a positive result was found for Thioflavin T in the gland cell, the reaction was not specific as the ovarian and connective tissues also fluoresced. The chemistry of the gland cells remained the same throughout the moulting cycle of the animal; therefore subsequent histochemical and immunostaining investigations were conducted without regard for moult stage.

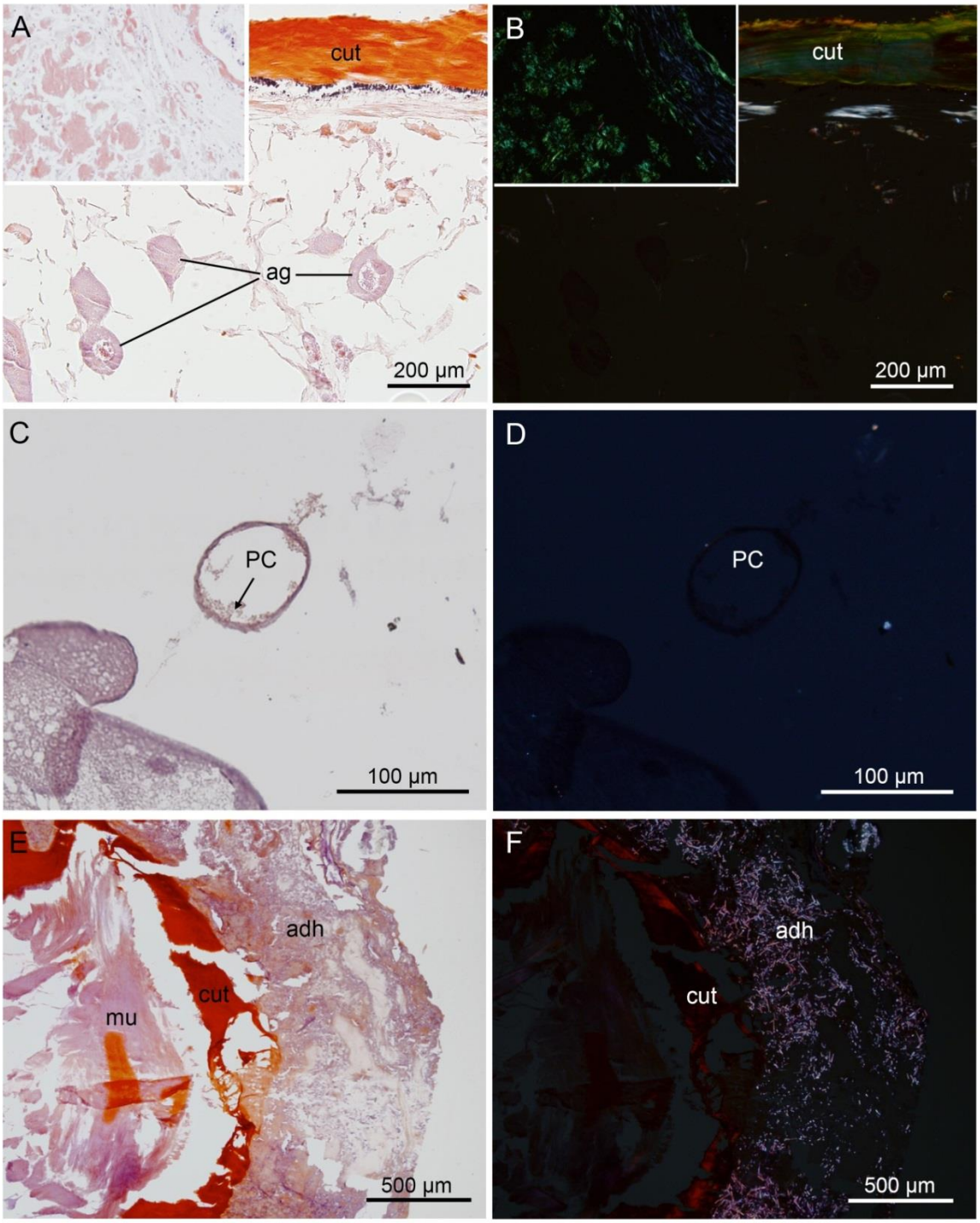

Figure 3.3: Congo red stain for amyloid under normal and cross-polarised light, showing an absence of amyloid (apple-green birefringent glow) in all barnacle tissues. A) Adhesive glands of *L. anatifera* under normal light and (B) cross polarised light. Insets: human kidney tissue with amyloidosis, showing a positive reaction under cross-polarised light. C) Principal canal with some secretion visible (arrow) under normal light and D) cross-polarised light. E) Cured adhesive alongside the epidermal cuticle under normal light and F) cross polarised light. Adh-cured adhesive, ag-adhesive gland, cut-epidermal cuticle, mu-muscle, PC-principal canal.

3.3.3 Assays for post-translational modifications

The adhesive glandular apparatus of *L. anatifera* was negative for both DOPA and pSer residues. The barnacle tissues did not react to the Arnow assay for DOPA, while the positive control (tubeworm) became bright red (later fading to yellow) throughout the adhesive gland tissue, confirming that the lack of reaction in the barnacle tissue was a true negative (Figure 3.4E). DOPA was not present in the adhesive gland cell, canal network or cured adhesive of *L. anatifera* (Figure 3.4A, C). After immunostaining for pSer residues only background staining was seen in the adhesive gland cells, canal network and cured adhesive of *L. anatifera* (Figure 3.4B, D), while the adhesive tissues of the tubeworm control stained darkly (Figure 3.4F). A strong positive reaction was seen beneath the surface of the peduncle, in the epidermal cells of *L. anatifera* (Figure 3.4D); this is to be expected as phosphorylated serines are known to be present in proteins that form the exoskeleton of crustaceans (Nagasawa, 2011).

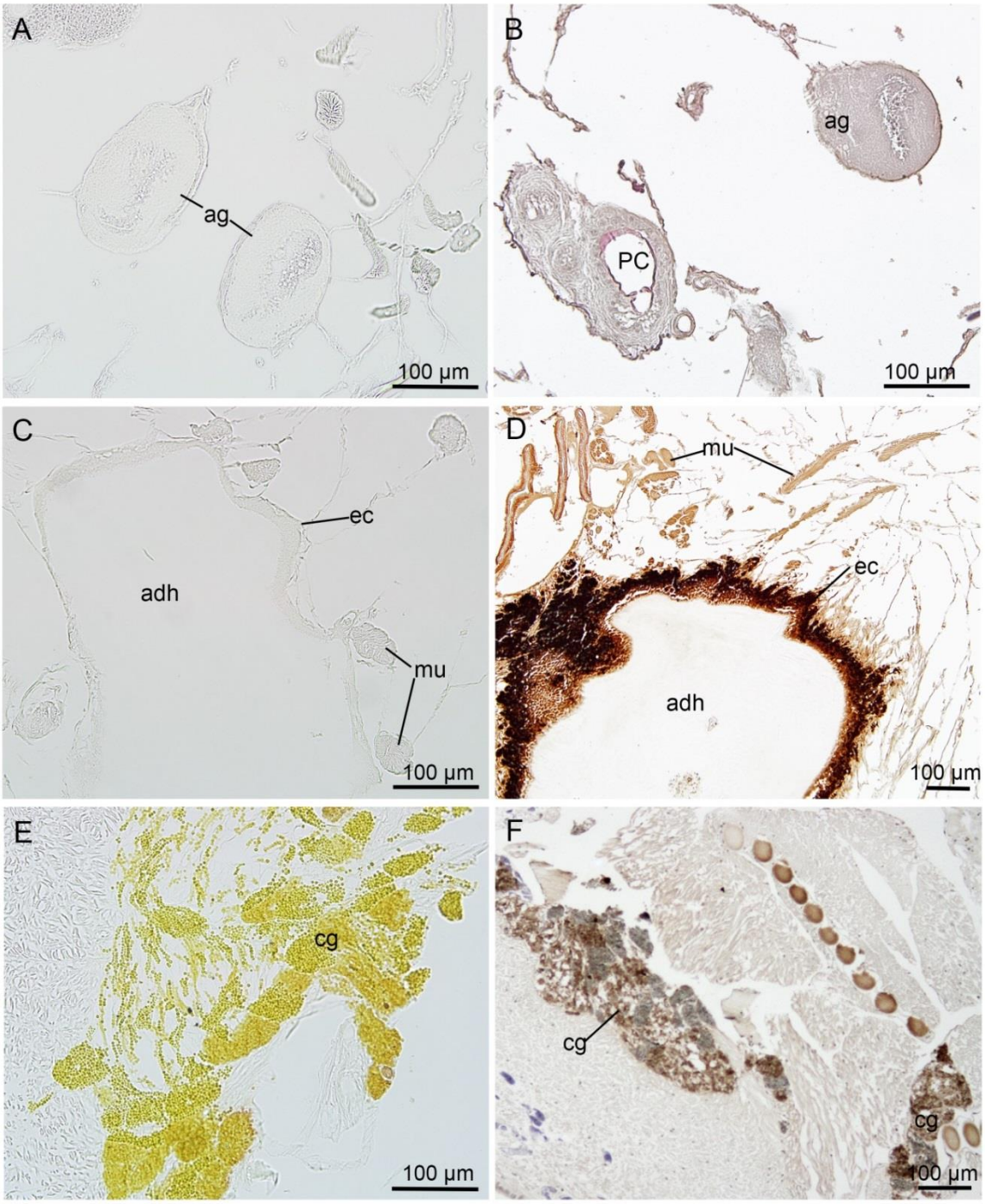

Figure 3.4: A) The adhesive gland cells of *L. anatifera* after the Arnow assay for DOPA, showing no reactivity. B) Adhesive gland and principal canal of *L. anatifera* after pSer immunostaining, showing no reactivity to the antibody, with slight background staining in all tissues produced by DAB. Staining of the PC lining was by the eosin counterstain. C) The hardened adhesive of *L. anatifera* at the base of the peduncle after Arnow assay, showing no reaction. D) Hardened adhesive after pSer immunostaining with background staining only. Reaction to pSer antibody can be seen in the epithelial cells underlying the exoskeleton of the peduncle while the colour seen in the muscle tissue is further background staining. E) Tubeworm *Sabellaria alveolata* cement glands showing positive reaction to the Arnow assay. F) Tubeworm cement glands showing positive reaction to pSer antibody. Adh-cured adhesive, ag-barnacle adhesive gland, cg-tubeworm cement glands, ec-epithelial cells, mu-muscle, PC-principal canal, arrows-tubeworm secretory granules.

3.3.4 Ultrastructure of the canal network

The intracellular canal (ICC) drains adhesive products from the gland cell to the collector canals (CCs) (Figure 3.5A and see below). The ICC was a large, lobed structure that extended along the outer cytoplasm, with extensions of ICC being visible at opposite sides of the cell (Figure 3.1C-E). In the ICC lumen, adhesive products were observed as dense material, usually homogenous but also seen as a loose, flocculent substance (Figure 3.5C, D, F). Narrow folds or processes of the ICC membrane were observed (Figure 3.5B-D), increasing the surface area of the ICC membrane and possibly enveloping portions of the gland cytoplasm. Secretory granules were closely associated with the ICC; these ranged from very electron dense to completely lucent granules, which fused to form larger structures of mottled appearance. Some secretory granules were observed to contain round or tubular structures and, more rarely, were seen to contain many small vesicles (Figure 3.5B-C). Secretory granules were seen in very high densities around the ICC and electron dense bands formed at the junctions between adjacent granules (Figure 3.5F). The cytoplasm immediately surrounding the granules was moderately electron dense and the membranes of what appeared to be granules were at times seen to be open; although these may have been either granules emptying their contents into the cytoplasm or small branches of the ICC enveloping the secretion (Figure 3.5B, C). The lumen of the ICC often contained multi-lamellate membrane bodies, possibly the remains of cytoplasmic structures from the glandular cytoplasm that were enveloped as the ICC formed.

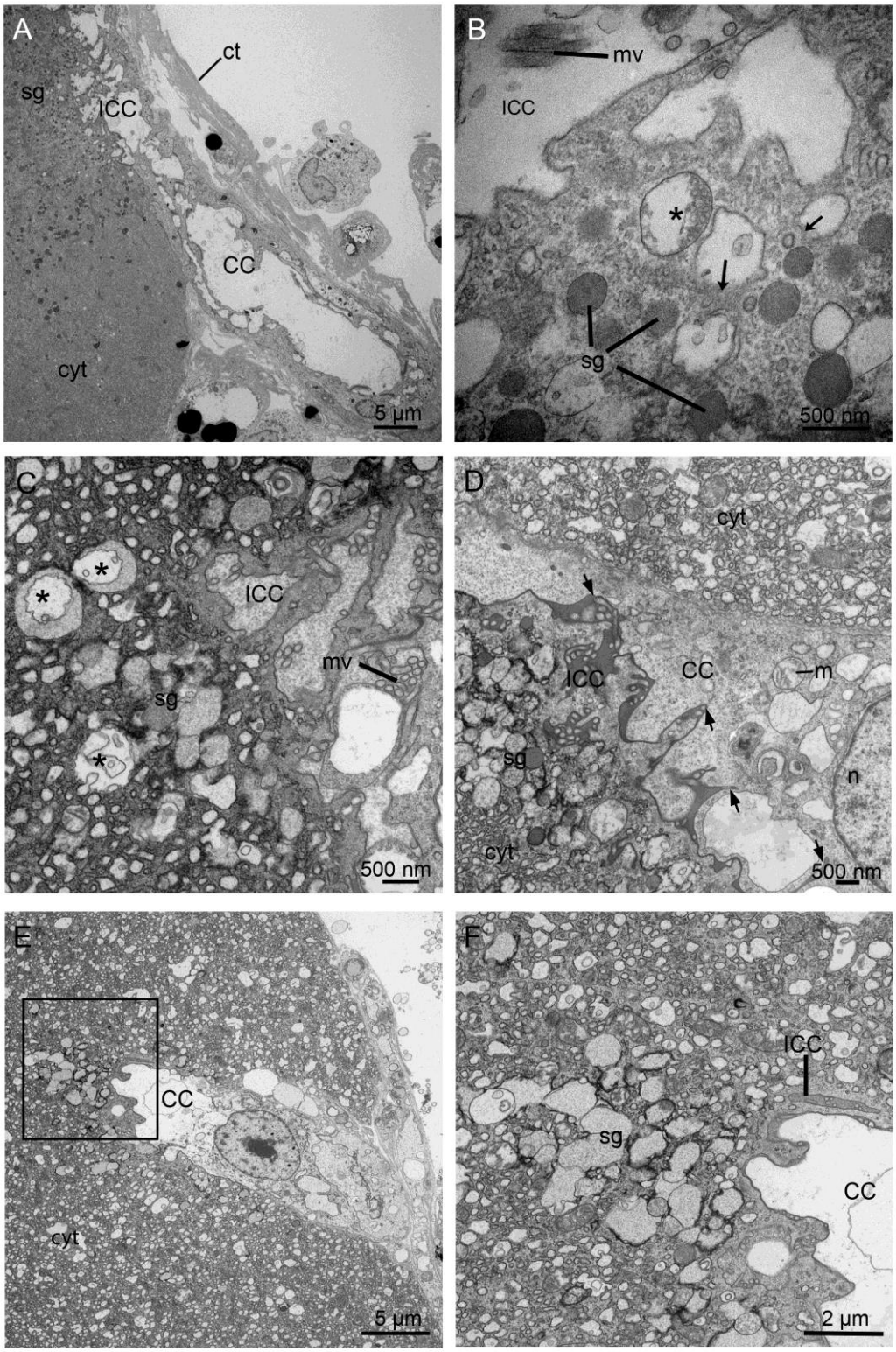

Figure 3.5: A) Adhesive gland cell with secretory granules in the cytoplasm and a well-developed ICC. B) Higher magnification of the ICC with microvilli-like processes, dense granules, lucent, partially membrane-bound structures (arrows) and vesicles containing small membrane-bound structures (asterisk). C) ICC with dense secretion and lucent pockets of loosely-packed material, with microvilli-like processes. Secretory granules surrounding the ICC are moderately dense, some with membrane-bound structures (asterisks). D) CC cell embedded in the gland cytoplasm. Branches of the ICC press deeply into the CC cell cytoplasm (arrows). E) Disintegrating CC cell pressed into gland cell cytoplasm. F) Higher magnification of E (indicated by black box) showing secretory granules adjacent to CC, with electron dense bands appearing where the granules meet. CC-collecting canal, ct-connective tissue, cyt-gland cell cytoplasm, ICC-intracellular canal, m-mitochondrion, mv-microvilli-like processes, n-nucleus, sg-secretory granules.

The appearance of the ICC within the cytoplasm varied from large spaces to thin branches that appeared to be enclosing sections of cytoplasm, indicating an apocrine process (Figure 3.5B, F). At times, as the secretory granules fused into larger structures, they became continuous with the ICC lumen, which is more indicative of merocrine exocytosis. Thus evidence for both of these secretory mechanisms was seen during adhesive drainage. Where the ICC occurred adjacent to CC cells, branches and processes of the ICC pressed deeply into the CC cell body (Figure 3.5D). The CC often had sections with a grainy flocculent substance, presumed to be adhesive, as this had the same appearance as the contents of the ICC.

Although histological observations showed the lumen of the CC to be continuous with the lumen of the secondary canal (SC), during ultrastructural observations cell membranes were always intact at this junction. The SC comprised of a lumen surrounded by a single layer of epithelial cells, which varied in shape depending on how well developed the canal was. While large, clearly defined cuboidal cells were seen around less developed SC, a squamous epithelium was visible around well developed, secretion-containing SC (Figure 3.6A, B). In some sections, the SC was still developing and in this case it had no clearly defined lumen; instead, the apical portions of the canal cells were empty of cytoplasmic structures and cytoplasm appeared to be degrading (Figure 3.6A). This degradation probably gave rise to the membrane-bound structures with the typical appearance of cytoplasmic remains that were often seen in the lumen of the SC.

3.3.5 Features of the principal canal and secretion

The principal canal (PC) had a diameter of 50 µm and had a thick, electron dense cuticle-like lining over the inner canal wall along its entire length (Figure 3.6C-G). The walls of the canals consisted of a single layer of epithelial cells which were of varying thicknesses. In the area of the ovary and gland cells, the PC epithelial cells were thick cuboidal, while in other locations they were thin and squamous. The secreted product of the gland cells was readily apparent within the lumen of the PCs, where the secretion accumulated at a greater density than in the smaller canals and became clumped together (Figure 3.6D-G). The secretion within the lumen was electron dense, stained

intensely for slightly acidic proteins (close to pH 6.0) and moderately for carbohydrates (Table 3.3).

Table 3.3: Histochemical analysis of the secretion seen within the principal canal of *L. anatifera*. Reaction defined as no reaction (-), weak reaction (+), moderate reaction (++) and strong reaction (+++).

Stain	Purpose	Reaction
Alcian blue pH 2.5 after HCl pretreatment	Acid mucopolysaccharides	–
Biebrich scarlet pH 6.0 after HCl pretreatment	Acid protein	+++
Congo red	Amyloid	–
Mercury bromophenol blue (MBPB)	General protein	+++
Benzoylation + MBPB	Control/block hydroxyl	++
PAS	Carbohydrate	++
Thioflavin T	Amyloid	+

The secretion and the hardened adhesive gave a negative result for amyloid with Congo red (Table 3.3; Figure 3.3C-F). Thioflavin T gave a positive result in the cuticle lining of the principal canal and weak fluorescence in the secretion, the ovary and connective tissue. The hard adhesive had a strong, uniform fluorescence. No histochemical reactions changed as the secretion passed down the peduncle to the adhesive base (Figure 3.6E-G). However, ultrastructural observations showed that the appearance of the secretion changed as it travelled further from the gland, from a loose, diffuse substance in the collecting and secondary canals to a denser, clumped substance in the principal canal. At no point were haemocytes observed in the secretion within the principal canals.

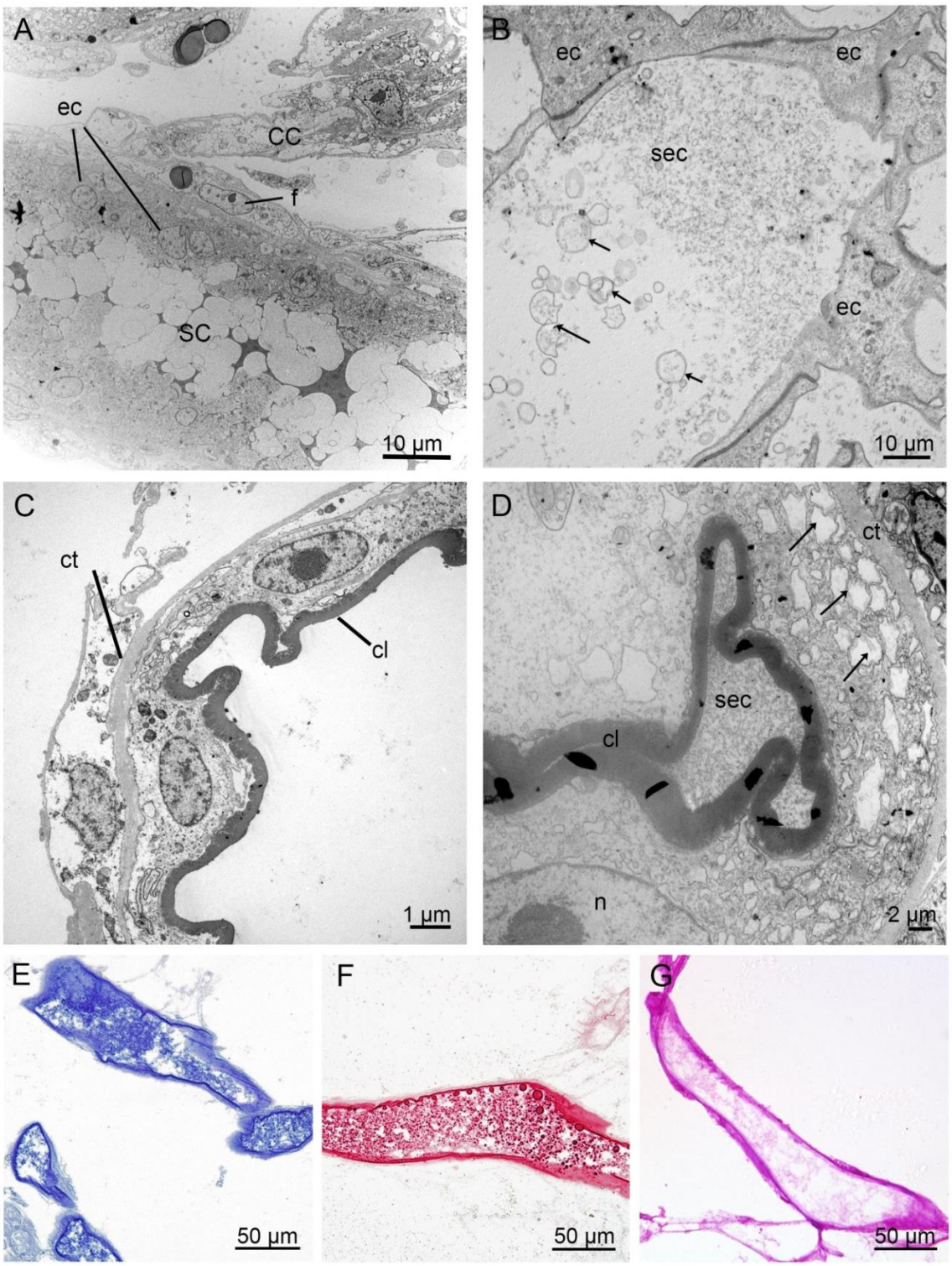

Figure 3.6: A) Formation of the canal network: the lumen of the SC is visible but remnants of cell breakdown remain and the epithelial cells that line the canal are large. The CC has not yet formed a lumen or become continuous with the SC. Fibroblasts can be seen in the connective tissue that lines the SC. B) Lumen of the SC with secreted adhesive and membrane-bound structures (arrows). C) PC with empty lumen and dense cuticle lining; dense connective tissue can be seen around the outside of the canal. D) PC with secretion in the lumen and large membrane bound structures in the surrounding epithelial cells (arrows). E) Bromophenol blue shows the secretion in principal canal to react positively for protein. F) Biebrich scarlet at pH 6.0 shows protein content to be in the acidic range. G) Periodic acid Schiffs reaction shows the presence of carbohydrate within the secretion. CC-collecting canal, cl-cuticle lining, ct-connective tissue, ec-epithelial cells, f-fibroblast, n-nucleus, SC-secondary canal, sec-secretion.

3.4 Discussion

We examined the morphology, chemistry and secretory pathway of the barnacle adhesive apparatus and found it to contrast sharply with what is observed in other well-studied adhering organisms. Unlike other adhesive models, the various components of barnacle adhesive were all produced from the same cell, where they were enclosed within cytoplasmic secretory granules. This has also recently been confirmed in the stalked barnacle *Dosima fascicularis* (Zheden et al., 2012). Besides granule formation, there was no physical separation of adhesive components at any stage of the secretory process; furthermore, once they had been released from the gland cell cytoplasm the adhesive components were completely unbound by granular membranes as they made their way through the adhesive drainage system. This is surprising because of the diversity of proteins believed to be involved in the barnacle adhesive complex and the risk of premature activation of curing in the adhesive. The secretory pathway was characterised by a large intracellular drainage canal, a dynamic structure which enveloped or fused with cytoplasmic secretory granules. The secretion of the proteins from the gland cell involved apocrine and merocrine secretion. Similar processes of envelopment, fusion and protein translocation potentially transport the adhesive from the intracellular canal into the extracellular environment of the collecting canal.

A marked contrast between adhesion in barnacles and other well-known organisms relates to the molecular mechanism involved: there was no evidence of the post-translationally modified amino acids DOPA or pSer in the adhesive gland or the cured adhesive. This confirms that the adhesive mechanisms common in mussels, tubeworms and a variety of other aquatic animals are not present in barnacles. The presence or absence of these PTMs in the barnacle adhesive system is unknown as many of the adhesive components remain uncharacterised. Our results showed that DOPA was not present in the gland cells or cured adhesive, however Lindner and Dooley (1972) reported the presence of phenolase and quinhydrone in adult glands cells, although phenols, including tyrosine, were not found in that study. Tyrosine, which is a precursor to DOPA when oxidised by an enzyme such as phenolase, has been observed histochemically in many other studies (Fyhn & Costlow, 1976; Karande & Gaonkar, 1977; Walker, 1970 and the present study), making DOPA formation theoretically possible. It now appears that the observation of phenolase in the gland cells by Lindner

and Dooley (1972) requires confirmation. Amongst the better-known gluing organisms, barnacles are therefore the only ones whose permanent adhesives do not contain these ubiquitous PTMs; DOPA has been observed in the adhesives of mussels and tubeworms (Jensen & Morse, 1988; Waite, 1983a) and pSers have also been found in mussels and tubeworms, as well as in sea cucumber cuvierian tubules, sea-star tube feet and in the freshwater caddisfly larval silk (Flammang et al., 2009; Hennebert, 2010; Stewart & Wang, 2010).

Histochemical analyses in the present study were an extension of previous work in acorn barnacles (Fyhn & Costlow, 1976; Karande & Gaonkar, 1977; Walker, 1970) and the first histochemical investigation into the adhesive glands of a stalked barnacle. They indicated that the proteins within the adhesive are slightly acidic and are rich in cystine, tyrosine, and hydroxyl groups in general (indicating the presence of serine and threonine). This was consistent with what is known regarding the amino acid sequences of barnacle proteins. The characterisation of three proteins from acorn barnacle adhesive have shown that cp-19k contains a large proportion of the amino acids serine and threonine, cp-20k has a large proportion of cystine and cp-100k has a relatively large amount of tyrosine compared to the others (Kamino et al., 2000; Mori et al., 2007; Urushida et al., 2007). Two further proteins do not apparently differ in major respects from those already characterised, although one is N-glycosylated (Kamino et al., 2012; Kamino & Shizuri, 1998). Recombinant forms of two of the aforementioned proteins, cp-19k and cp-20k, have been achieved and these proteins retained their functionality with respect to adhesion on various surfaces (Mori et al., 2007; Urushida et al., 2007). Our preliminary results had indicated that there were highly acidic substances within the gland cell (Power et al., 2010), however further histochemical investigations showed that this result was likely due to high amounts of RNA in the cytoplasm, as is to be expected from cells producing large amounts of proteins. There was no histochemical evidence of extremely basic and extremely acidic proteins (with corresponding opposite charges) in the adhesive gland or hardened adhesive of the barnacle; the gland stained at pH 3.5-6 with a stronger reaction at the pH 6 end of the range. The isoelectric points (pI) of the barnacle adhesive proteins reported by others are variable; for example, cp-19k and cp-20k have pI values of 5.8 and 4.72 in *Megabalanus rosa*, but pI values of 10.3 and 8.3 in *Fistulobalanus albicostatus* and *Amphibalanus improvises,* respectively (Kamino & Shizuri, 1998; Mori et al., 2007; Urushida et al., 2007). It is therefore highly

probable that the adhesive proteins in *L. anatifera* differ in pI to those of acorn barnacles, with the bulk of the adhesive in *L. anatifera* comprising of proteins with moderate pI values.

The proportion of cysteine in the whole adhesive of acorn barnacles has similarly varied between studies with detection ranging from approximately 16 to 90 residues per thousand (Kamino et al., 1996; Naldrett, 1993). Results of the present study show that the cysteine residues at the point of synthesis within the barnacle gland are predominantly present as dimers, which indicates that disulfide bonds potentially play some role in the barnacle adhesive. The importance of disulfide bonds is also indicated by the utility of reductants in solubilising the adhesive complex (Naldrett, 1993; Naldrett & Kaplan, 1997). This issue is, however, unresolved, as it has recently been reported that disulfide bonds are only important as an intramolecular force conferring 'shape' rather than as a major cross-linking force (Kamino et al., 2012). Disulfide signatures were also absent from Raman spectra of freeze-dried adhesive and non-covalent bonding such as hydrophobic and electrostatic interactions were suggested to be important instead (Wiegemann et al., 2006). Saw-tooth signatures in atomic force measurements as well as staining indicative of amyloid fibres have previously been seen in cured *Amphibalanus amphitrite* adhesive (Barlow et al., 2010; Sullan et al., 2009) and amyloid-like β-sheet formation has been predicted from sequence data for the large adhesive protein cp-100k (Kamino et al., 2000). Thioflavin T is routinely used as well as Congo red to confirm the presence of amyloid but this gave ambiguous results in the present study; a strong, unspecific reaction was seen in various tissues. The specificity of Thioflavin T for amyloid is questionable (Kelienyi, 1967; Khurana et al., 2005), however Congo red is a definitive, diagnostic assay. Congo red showed a negative result for amyloid in the glands, adhesive secretion and cured adhesive of *Lepas anatifera*, in contrast to approximately 5% amyloid previously observed using a similar staining technique on cured *A. amphitrite* adhesive (Sullan et al., 2009). It is possible, but unlikely, that this discrepancy is due to species differences; the precise involvement and role of amyloid requires more work.

While carbohydrate was observed within the gland cell, this could have been unrelated to the adhesive. However, the presence of carbohydrates in the secretion (within the canals) showed that carbohydrates are definitely a component of the adhesive of *L.*

anatifera. The carbohydrate content of the adhesive gland of a related species, *Dosima fascicularis*, has been further defined; histochemical tests showed that the gland cell contained sulfated carbohydrates at pH 4.5-5.5 (McEvilly, 2011). Sugars (glycoproteins and proteoglycans) are important molecular components in the temporary adhesives of echinoderms, cephalopods and gastropods (DeMoor et al., 2003; Hennebert et al., 2011; Smith, 2010; von Byern & Klepal, 2006) but they have not been considered important in the permanent adhesives of barnacles, mussels and tubeworms. Previous investigations into the biochemical composition of the entire barnacle adhesive have consistently returned carbohydrate values of less than 3% (Barnes & Blackstock, 1974; Walker, 1972; Walker & Youngson, 1975). The recent characterisation of cp-52k in *M. rosa* has shown N-glycosylation to be present, though the functional significance of this is yet to be examined (Kamino et al., 2012).

The adhesive systems of the mussel and tubeworm involve more than one cell type producing distinct secretory materials. The tubeworm adhesive is produced by multiple cells in the building organ and parapodia which produce at least four types of granule, prominently seen at a histological level (Wang & Stewart, 2012). These remain intact and separated from each other after secretion from the cell, until they reach the level of the building organ where the adhesive is released (Wang et al., 2010). Several distinct granule types can be distinguished in the mussel adhesive system, which are localised in different parts of a multi-glandular system: the phenol gland has dense round homogenous granules, the enzyme gland has mottled granules and the collagen gland ellipsoidal granules containing filaments (Tamarin & Keller, 1972). The distinctive granular morphologies reflect the distinct components that are produced in different areas; they do not mix until they are secreted into the ventral groove of the foot, which serves as a ‘mould’ for the byssal thread (Tamarin & Keller, 1972). The morphology of both systems appears to help regulate the secretion of components which, if mixed at the point of synthesis, would polymerise prematurely. This regulation is further boosted by the involvement of enzyme catalysis of the adhesive (Waite, 1992; Wang & Stewart, 2012). Interestingly, this type of system is also seen in larval barnacles, in the cyprid stage, which forms the initial attachment to the substrate. Barnacle cyprids are known to also possess a multicellular adhesive gland, containing two different cell-types, each associated with distinctive adhesive-containing granules (Okano et al., 1998; Walker, 1971). In all cases, these multi-glandular adhesive systems facilitate controlled secretion

and self-assembly of adhesive components, often with an enzyme added at the point of release to catalyse the curing process.

In sharp contrast with each of the cases outlined above, less organisation is apparent in the adult barnacle adhesive system, where only one large gland cell type is involved. Granules were much smaller in barnacles than in other adhesive animal models; in *L. anatifera* glandular tissue they were only visible at the ultrastructural level. Granules were no longer bound after secretion into the intracellular canal, implying that the various components begin to be mixed within the gland cell. Therefore, interactions between the proteins and self-assembly of the adhesive may be a slow process in the barnacle, as the adhesive must remain soluble while within the animal. In the present study the fundamental chemistry of the *L. anatifera* adhesive did not change at a basic histochemical level as it travelled along the length of the drainage system, indicating that very little changes to the adhesive occur while it remains inside the body. It was first suggested by Naldrett (1993) that the adhesive components were likely to begin interacting with each other long before being released from the animal, while more recently Kamino et al. (2012) reiterated that the adhesive of adult barnacles is not necessarily rapid-setting; the barnacle is not creating an attachment but supplementing the adhesive that is already present, thus the freshly released adhesive has time to cure and adhere slowly. While it has been reported that the adhesive takes several hours to fully solidify (Cheung et al., 1977), the time elapsed between the synthesis of proteins and the release of the adhesive is unknown. It is unlikely that the curing process of the barnacle adhesive is dependent on time alone, although a mechanism to catalyse the process has not yet been found. There has been no evidence of enzymatic activation of barnacle adhesive to date and the adhesive apparently polymerises in air as well as in salt water, thus pH or osmolarity may not be significant in triggering the adherence and setting of the adhesive (Cheung et al., 1977; Dougherty, 1996; Kamino, 2006, 2010b). In contrast to this, a synthetic peptide based on repetitive sequences from barnacle cp-20k (with some slight modifications) responds to changes in salt concentration or pH by self-assembling into a membrane of interwove filaments or a three-dimensional mesh-like structure with defined pores (Nakano et al., 2007). A possible colloidal system involving emulsifiers or protecting colloids to keep components in a thermodynamically pseudo-stable state has been suggested but this has not yet been tested (Wiegemann, 2005).

The observations made in this study draw attention to the ambiguity that still surrounds the molecular basis of barnacle adhesion. For example, it has been proposed that the curing of the adhesive is related to the wound healing process and utilises transglutaminase from haemocytes (Dickinson et al., 2009). The production of transglutaminase in invertebrates is generally considered to originate in the haemocytes, although other sources of transglutaminase may include muscle tissue, the gill and the lymphoid organ, as has been seen in shrimp and scallops (Liu et al., 2007; Nozawa et al., 1997). The current study found no structural evidence for the involvement of haemocytes in the adhesive: no haemocytes were seen anywhere within the adhesive system of the barnacle, including the secretion, and there was no point of entry to the adhesive system for haemocytes. If the methods used by Dickinson et al. (2009) to harvest liquid adhesive had damaged the base of the animal, haemolymph would have been released, providing a possible source of transglutaminase in that study (Kamino, 2010b; Power et al., 2010). Barnacle haemolymph may actually contain few haemocytes, whose primary role is phagocytising bacterial cells not the coagulation of haemolymph to form a clot (Waite & Walker, 1988).

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4 Scanning electron microscopy and spectroscopic analyses of the adhesive of *Lepas anatifera*

4.1 Introduction

Barnacle adhesion retains an air of mystery in the field of adhesive biomaterials; despite a wide range of recent studies, relatively little is known about the molecular mechanism that allows permanent attachment underwater. Recent investigations have included characterisation of adhesive proteins, predictions of their secondary structure, examinations of the proteins' adhesive properties to various materials, the ultrastructure of the adhesive system and the mechanical properties of the adhesive (e.g. Jonker et al., 2012; Kamino et al., 2012; Raman & Kumar, 2011; Sullan et al., 2009; Suzuki et al., 2006; Zheden et al., 2012). Amongst other things, these have shown that the barnacle adhesive system does not utilise either of the more common molecular adhesive mechanisms: phosphorylated serines (pSer) or L-3,4-dihydroxyphenylalanine (DOPA) (Jonker et al., 2012; Kamino et al., 1996), which in turn raises the possibility of finding adhesive novelties in the barnacle model.

Elemental analyses combined with structural investigations could provide a deeper understanding of the barnacle adhesive, as it has done for caddisfly (Stewart & Wang, 2010), tubeworm (Stewart et al., 2004) and mollusc adhesives (Werneke et al., 2007). In these organisms, elemental analyses provided important clues; for example, the tubeworm adhesive was shown to have high phosphorus content, leading to the discovery of the non-standard amino acid pSer in the adhesive (Stewart et al., 2004). Similarly, the observation of transition metals (including zinc, iron, copper and manganese) in mollusc adhesive gels eventually led to the discovery that transition metals have an essential stiffening effect on the gel, possibly via catalysis or co-ordination of cross-linking (Smith et al., 1999; Werneke et al., 2007). Elemental investigations have highlighted iron-DOPA complexes, which have been shown in mussel adhesion (e.g., Hwang et al., 2010b) and tubeworm adhesive complexes with calcium and magnesium ions (Stewart et al., 2004). However, information for metal/inorganic involvement in the adhesive mechanism is generally lacking for barnacles and there have been only two reports of scanning electron microscopy (SEM) with energy dispersive spectrometry (EDS) data for barnacle adhesive (Berglin &

Gatenholm, 2003; Sullan et al., 2009). The benefits of SEM-EDS compared to methods that are more sensitive or quantitatively precise is that the elements can be mapped *in situ* within the gland or adhesive interface.

Optical spectroscopy can also be used to understand chemical composition, molecular bonds and molecular conformation in complex biological molecules like proteins (Schweitzer-Stenner, 2001; Zhu et al., 2011). Using spectroscopy to examine biological adhesives is a relatively recent development (see review by Barlow & Wahl, 2012) but such methods have already provided fresh insights into mussel adhesive mechanisms. For example, Fourier transform infrared (FTIR) spectroscopy was used to explore DOPA-iron bonds in mussel byssus and adhesive (Sever et al., 2004). This method highlighted alternative bonding mechanisms, including enzyme activated di-DOPA complexes and DOPA-copper complexes for mussel adhesion (Fant et al., 2010). Meanwhile, Raman spectroscopy was used to investigate iron binding to DOPA, in the entire adhesive plaque and in purified foot-protein (fp) -1 and fp-2 (Hwang et al., 2010b; Taylor et al., 1996). Previous FTIR studies of barnacle adhesive have revealed the presence of amyloid-like β-sheet structures and phenolic compounds (Barlow et al., 2010; Burden et al., 2012).

The optical methods described above each have strengths and weaknesses. The FTIR spectra of proteins are dominated by the amide bands arising from the primary structure (protein backbone) and are not generally used to deduce specific amino acid side chain vibrations, although they are sensitive to sulfated and phosphorylated forms of biological molecules and phenolic groups (Barlow & Wahl, 2012). In contrast, Raman spectra of proteins contain the Amide I, II and III peaks, which reflect the common backbone of all amino acids, as well as peaks characteristic of amino acid side chains; the side chains of aromatic amino acids in particular give intensive Raman peaks (Dollish et al., 1974). Raman spectroscopy produces well defined bands compared to infrared spectroscopy methods and in recent years has been gaining momentum in biological applications (De Gelder et al., 2007). However, for barnacle adhesive, Raman spectroscopy has thus far only been used once, to rule out any significant presence of disulfide bonds (Wiegemann et al., 2006).

The current study aims to investigate the adhesion of the stalked barnacle *Lepas anatifera*. Examining this species will add information about a new taxon of barnacles with contrasting characteristics to the acorn barnacle species with calcareous bases, which have been the models used for almost all of the previous investigations. The latter connects two hard surfaces using an adhesive that is generally produced as a very thin, almost invisible layer. The stalked barnacles of order Lepadiformes have a very different morphology to acorn barnacles and their adhesive plaque is larger and formed at the tip of the relatively soft, flexible stalk. The small size of acorn barnacles and presence of a calcareous base plate complicates the conclusions drawn from adhesive studies using these barnacles, whereas the stalk and membranous base of *L. anatifera* allows results to be gathered with no interference from the base or lateral shell plates. The specific aims of this study were to investigate the structural, elemental and chemical properties of *L. anatifera* adhesive using SEM, EDS, Raman spectroscopy and FTIR spectroscopy, and to compare the results to what is known from acorn barnacle adhesive. SEM and EDS were also used to examine the adhesive gland cells, a method that has not previously been applied in barnacle adhesive research.

4.2 Materials and methods

4.2.1 Sample collection

Samples of live *L. anatifera* were collected opportunistically from the wild. Substrates included painted metal (data buoys), glass, plastic and nylon rope. *L. anatifera* was observed to produce a thick, opaque adhesive plaque on all of these substrates, similar to what is generally described as secondary adhesive in acorn barnacle species (Figure 4.1). The adhesive generally had a rubbery consistency and small pieces could easily be pulled away from the cuticle of the barnacle with sterile forceps. Where possible, large pieces of adhesive were carefully removed from inside the adhesive plaque, which is located at the base of the peduncle. This was carried out using a clean razor blade and the surfaces that had been exposed to the outside environment were cut away. Clean adhesive was then placed directly into eppendorf tubes, stored at -70 °C and subsequently freeze-dried at -50 °C (Labconco Stoppering Tray Dryer) and stored in a desiccator.

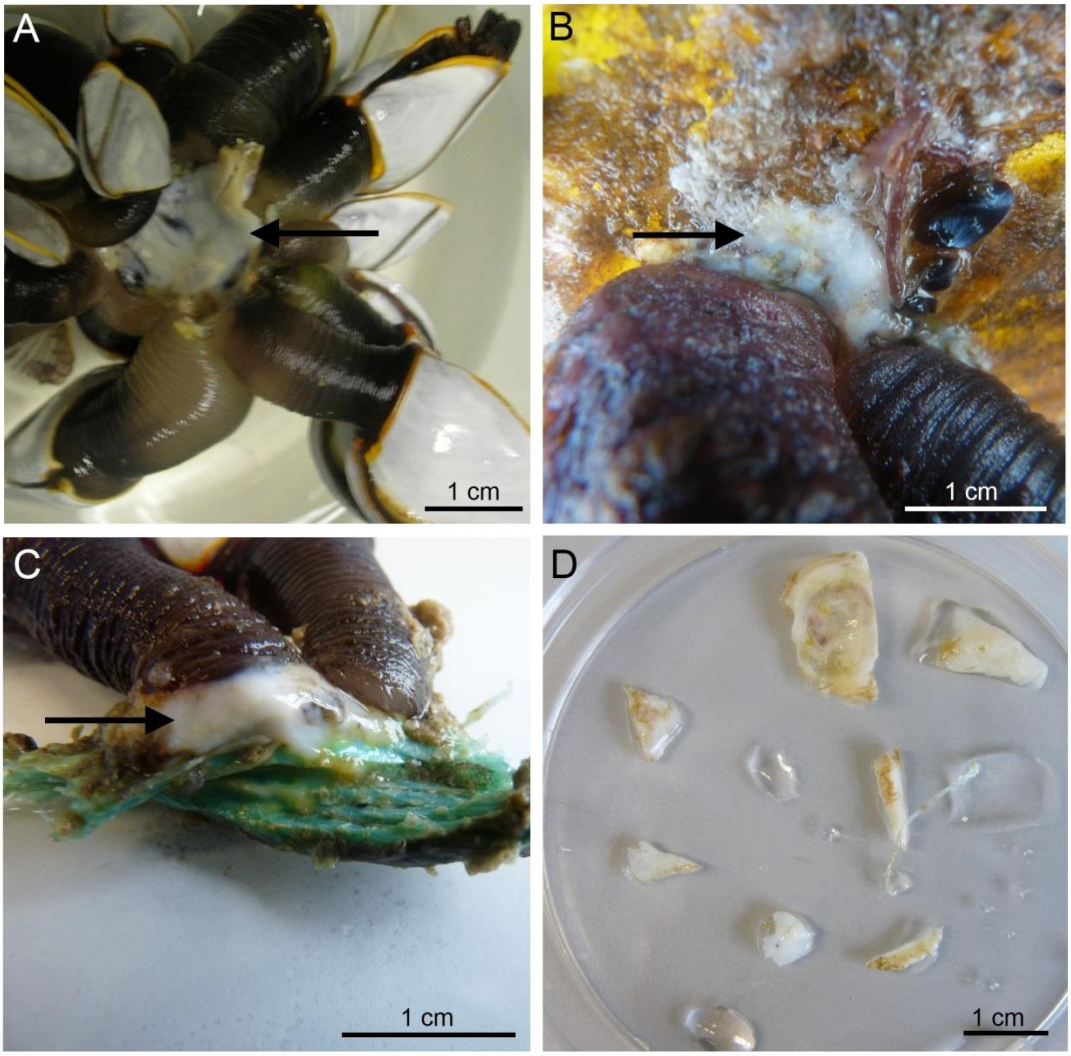

Figure 4.1: Appearance of the adhesive of *Lepas anatifera* on various substrates is indicated by arrows. A) *L. anatifera* that had been adhered to glass, with opaque white adhesive plaque visible at the centre of the group of individuals; B) *L. anatifera* adhered to a metal buoy; C) *L. anatifera* adhered to nylon rope; D) adhesive after removal from *L. anatifera*.

4.2.2 SEM-EDS

Four specimens of *L. anatifera* were fixed in Carnoy's fixative (Kiernan, 2008) and dehydrated in 70% alcohol before being sectioned by hand using a sharp razor blade. Sections were initially viewed under a stereomicroscope (Olympus SZX16) in order to select suitable sites for further study, then air-dried and gold-coated for SEM. Freeze-dried samples of adhesive from six different specimens of *L. anatifera* were also gold-coated for SEM. Investigations of the fine structure and chemistry of the adhesive glands and adhesive were performed using SEM in backscatter and secondary emission mode (Hitachi S-4700, acceleration voltage 20 kV, emission current 10 µA, working distance 12 mm). Spatial elemental distribution mapping using energy dispersive

spectrometry (EDS) (INCA, Oxford Instruments) was carried out to determine the presence of a range of elements. For both dehydrated tissue sections containing adhesive gland cells and freeze-dried adhesive samples, EDS spectra were collected as area scans, point scans and elemental maps.

4.2.3 TEM-EDS

Sample preparation for TEM was carried out as previously described (chapter 3; Jonker et al., 2012). Sections were cut at thicknesses ranging from <100 nm to 500 nm and mounted on formvar coated grids. Ultrathin (<100 nm) sections were used to create a 'map' of structures in each sample (Hitachi H7000); these were used as a reference when thicker sections (200 nm) were viewed using the TEM. The latter was a JEOL JEM-2100F instrument, which was used in STEM mode at 200 kV to obtain bright field (BF) and high angle annular dark field (HAADF) images, as well as EDS spectra

4.2.4 Raman spectroscopy

Freeze-dried *L. anatifera* adhesive was prepared from four different specimens and placed on clean glass slides. Raman spectra at random points were obtained using a Horiba LabRam HR confocal Raman microscope at 785 nm laser excitation. Suitable signal to noise ratio were generated using two accumulations over 400-3200 cm^{-1} (spectral resolution 1 cm^{-1}). Interference correction software was used to correct for and reduce the effects of fluorescence and remove artefacts caused by remaining background fluorescence.

4.2.5 FTIR spectroscopy

FTIR was used to analyse freeze-dried *L. anatifera* adhesive from four different specimens. The samples were not subjected to deuterium oxide (D_2O) treatment as barnacle adhesive is highly insoluble and cannot be brought into solution without denaturants and reductants, which would have affected the FTIR spectra (Kong & Yu, 2007). FTIR spectra were obtained using a Shimadzu FTIR-8300 at 4 cm^{-1} resolution between 600 and 4000 cm^{-1} (20 scans per sample). Background measurements were taken before each scan to create a baseline for the spectra.

4.3 Results

4.3.1 SEM of peduncle tissues

Prior to SEM, the sections were first viewed under a stereomicroscope while still immersed in ethanol. They were subsequently viewed in environmental backscatter mode. The transition from light microscopy to electron microscopy allowed the various structures to be easily located and identified, including adhesive gland cells (Figure 4.2). Secondary emission SEM mode showed that adhesive gland cells were large and located at the periphery of the peduncle, amidst strands of connective tissue. Unlike the ovarian tubules, which are packed tightly together, each adhesive gland was observed to be independent of the others. The surface of the adhesive glands was smooth; the contents of some adhesive glands were exposed due to rupturing during processing and contained many spherical globular components (Figure 4.2D). The contents of the adhesive glands ranged from being tightly packed with spherical globules to being loosely packed with a mixture of spherical globules and amorphous, porous globules.

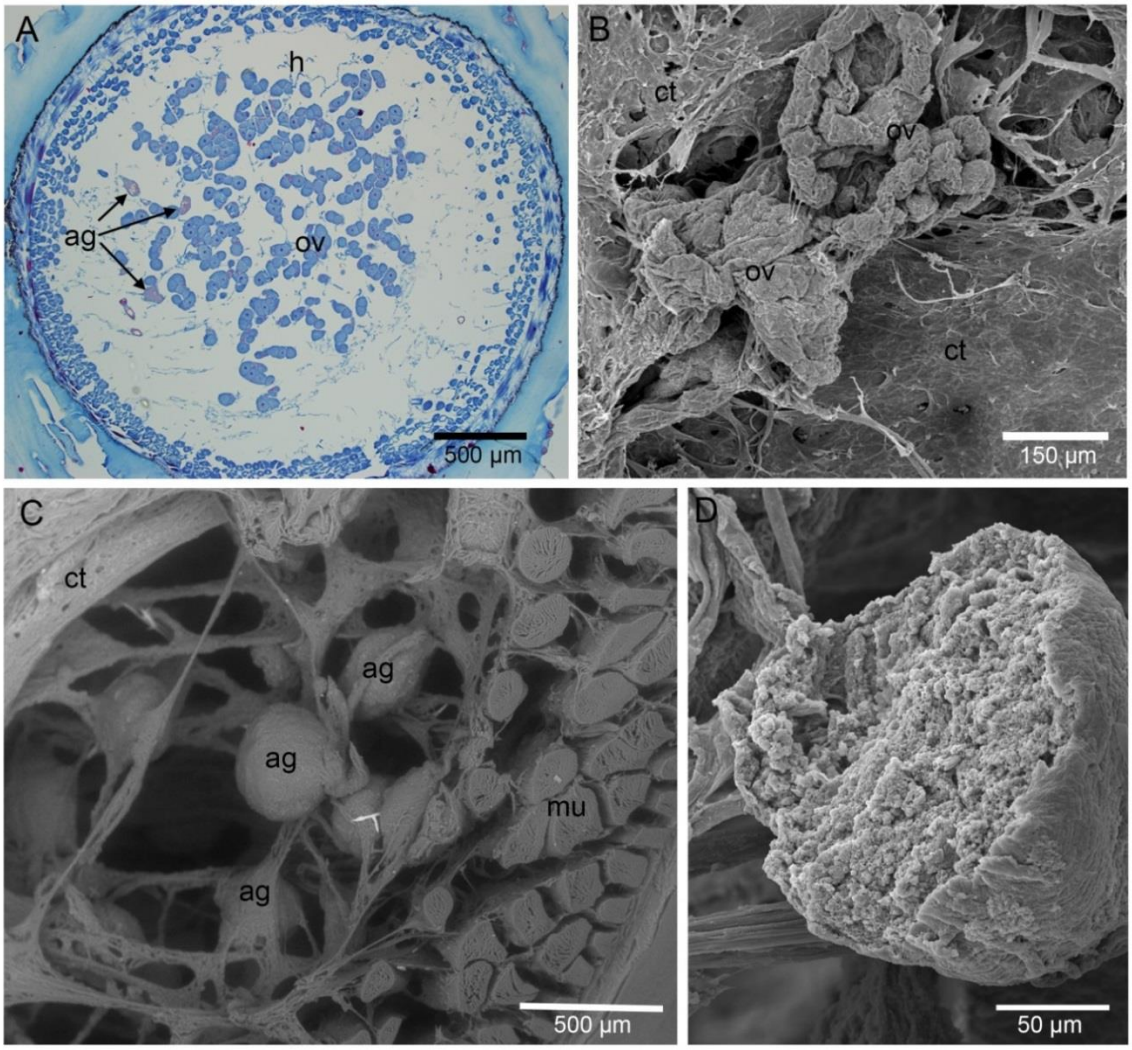

Figure 4.2: A) Light microscope image of transverse section through *L. anatifera* peduncle, stained with Azan; B) SEM image of the ovarian tubules and connective tissue; C) SEM image of the adhesive glands, muscle and connective tissue; D) High magnification view of a ruptured adhesive gland cell, showing a relatively smooth surface and tightly packed globular contents. Ag-adhesive glands, ct-connective tissue, h-haemolymph vessel, mu-muscle, ov-ovarian tissue.

4.3.2 SEM-EDS of adhesive gland

Elements detected consistently in all gland samples were carbon (C), oxygen (O), nitrogen (N), sodium (Na) and magnesium (Mg). Other elements were more variable; in some samples calcium (Ca), chlorine (Cl) or phosphorus (P) were also detected. Elemental mapping of C, O, Na and Mg appeared to reflect the structures observed in the sample (Figure 4.3C), however P and Ca were distributed ubiquitously across the field of view. There was no apparent distinction between cell surface and cell contents; all detectable elements were present across the entire area examined, however this may have been due to the rupturing of cells during tissue processing.

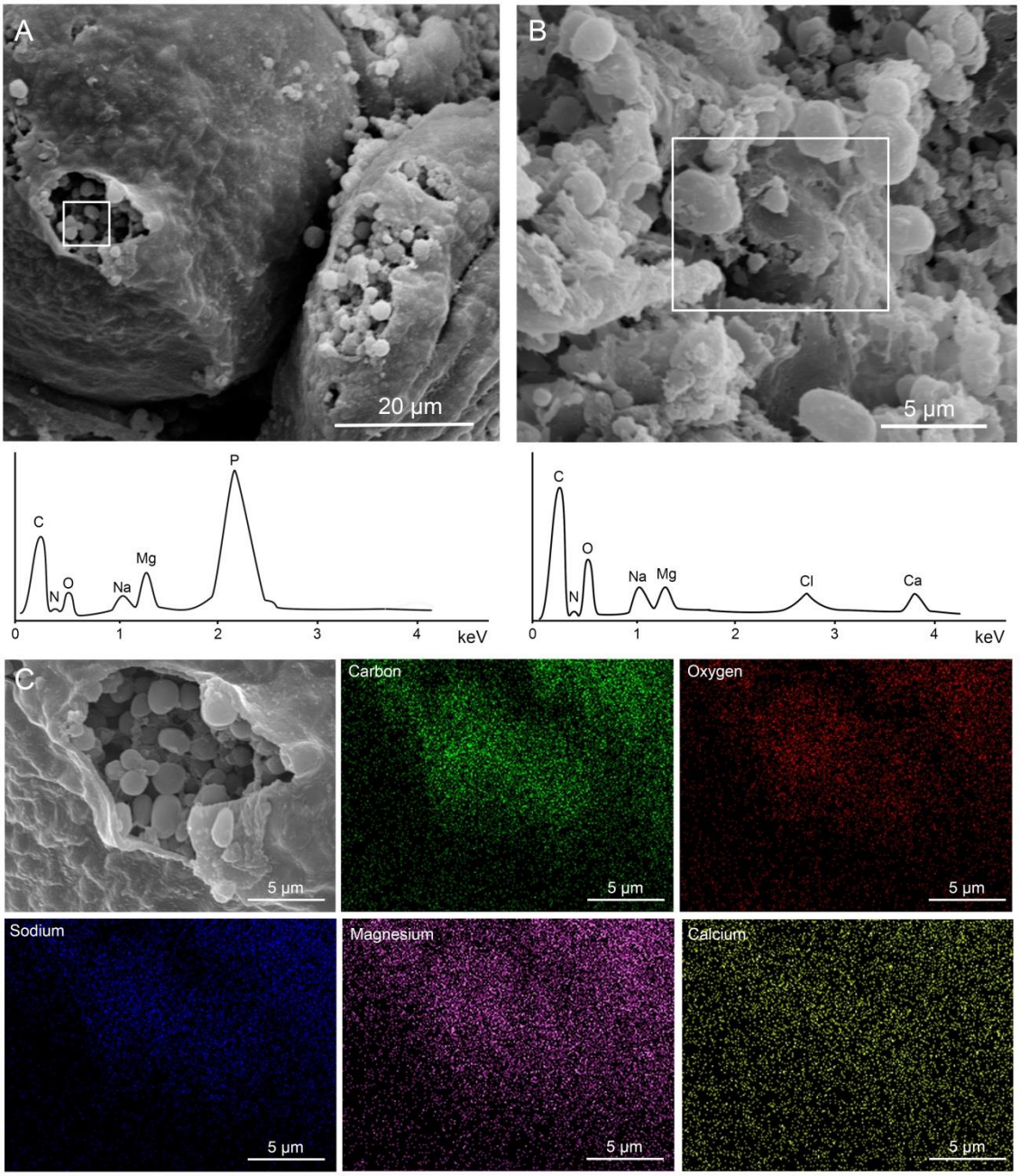

Figure 4.3: SEM-EDS of *L anatifera* adhesive glands: A) two adhesive glands with contents visible, box indicates location of area scan corresponding to EDS spectra. B) High magnification of adhesive gland cell contents from a different specimen, box indicates location of area scan corresponding to EDS graph. C) EDS maps (same specimen as A) showing distribution of carbon, oxygen, sodium, magnesium and calcium.

4.3.3 SEM-EDS of barnacle adhesive

SEM-EDS was used to investigate the elemental characteristics of freeze-dried barnacle adhesive. The adhesive examined was not interfacial; it was collected from within the bulk of the adhesive and thus less likely to be contaminated with impurities from the substrate. The elements detected in the adhesive were far more varied than what was observed in the glands (Figure 4.4). The elemental constituents of the protein backbone

(C, N, O), plus the additional elements commonly seen in the adhesive glands (Na, Mg, Ca, Cl) were detected in most of the samples. In addition, all adhesive samples contained sulfur (S), which was not detected in the adhesive glands. Some adhesive samples also contained aluminium (Al), silicon (Si), potassium (K) and iron (Fe); however P was detected only once (from a total of 44 EDS scans). The appearance of the adhesive (which had been taken from within the bulk of the adhesive plaque) varied within individual samples; surfaces ranged from smooth with an overlying fibrous network to porous in texture.

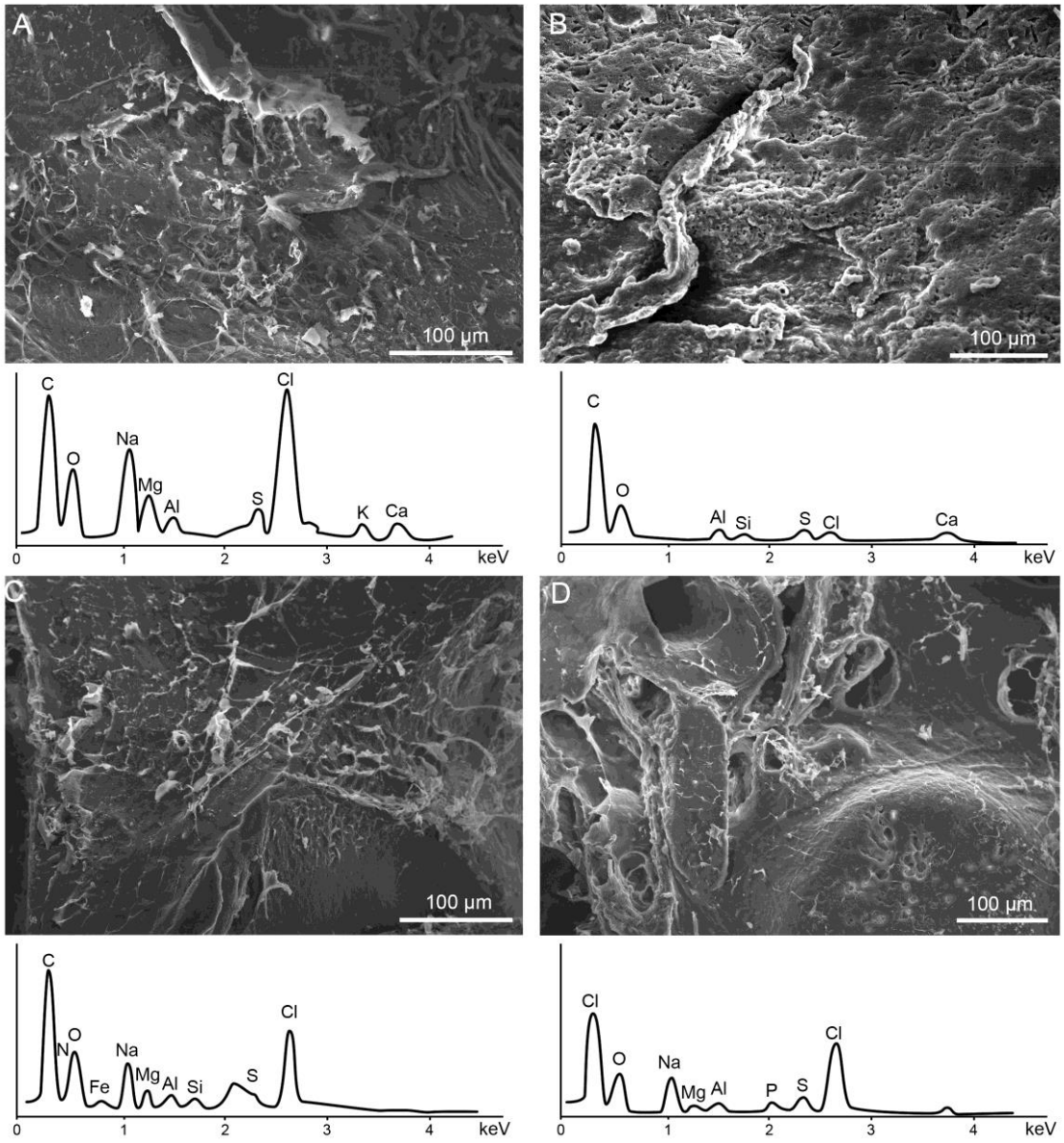

Figure 4.4: SEM micrographs of *L. anatifera* adhesive with corresponding EDS data from area scans of entire field of view for each image: A) surface of adhesive is covered by a network of fibres; B) surface of adhesive is porous and globular; C) surface of adhesive is primarily covered by a network of fibres, or is a smooth surface with no fibres (as observed in bottom right corner of image); D) surface of adhesive is interrupted by large pores (top left) and small pores (bottom right), with some fibrous structures.

4.3.4 TEM-EDS of peduncle tissues

In addition to examining the adhesive gland cells under SEM, attempts were made to examine thin sections using TEM with EDS. No distinctive elemental signal could be observed from the tissue compared with the empty resin. Copper and oxygen were detected but were concluded to originate from the formvar-coated grid.

4.3.5 Raman spectra of barnacle adhesive

Four biological replicate samples of freeze-dried adhesive from different specimens of *L. anatifera* were subjected to Raman spectroscopic analysis and very similar spectra were returned for all samples (Figure 4.5). The strongest peak, at 1002 cm^{-1}, was due to the phenylalanine (Phe) ring. Strong peaks also arose from a marked protein fingerprint, in the range of the Amide I band (primarily C=O bonds, at 1630-1680 cm^{-1}) and the Amide II band (primarily N–H, some C–N, at 1440-1490 cm^{-1}), with weaker peaks in the range of the Amide III band (mostly C–N, some N–H, at 1250-1350 cm^{-1}). Peaks with a Raman shift from 2800-3100 were also very striking; these were due to C–H bonds arising from the protein backbone (Movasaghi et al., 2007). The broad, weak peaks at the low end of the scale may also be due to small contributions from lipid (~415 cm^{-1}) (Movasaghi et al., 2007).

Many peaks were present between 600 and 1700 cm^{-1}, which were assigned to various amino acid side chains (Table 4.2). Phosphate groups produce strong peaks at 860 and 970 cm^{-1} (Movasaghi et al., 2007), however, Raman spectra of *L. anatifera* adhesive indicated a lack of phosphate groups. Further peaks that were noticeably lacking in the *L. anatifera* adhesive Raman spectra were protein-metal interactions, which in mussel byssus result in strong peaks dominating the spectrum between 400 and 650 cm^{-1} (Harrington et al., 2010; Hwang et al., 2010b).

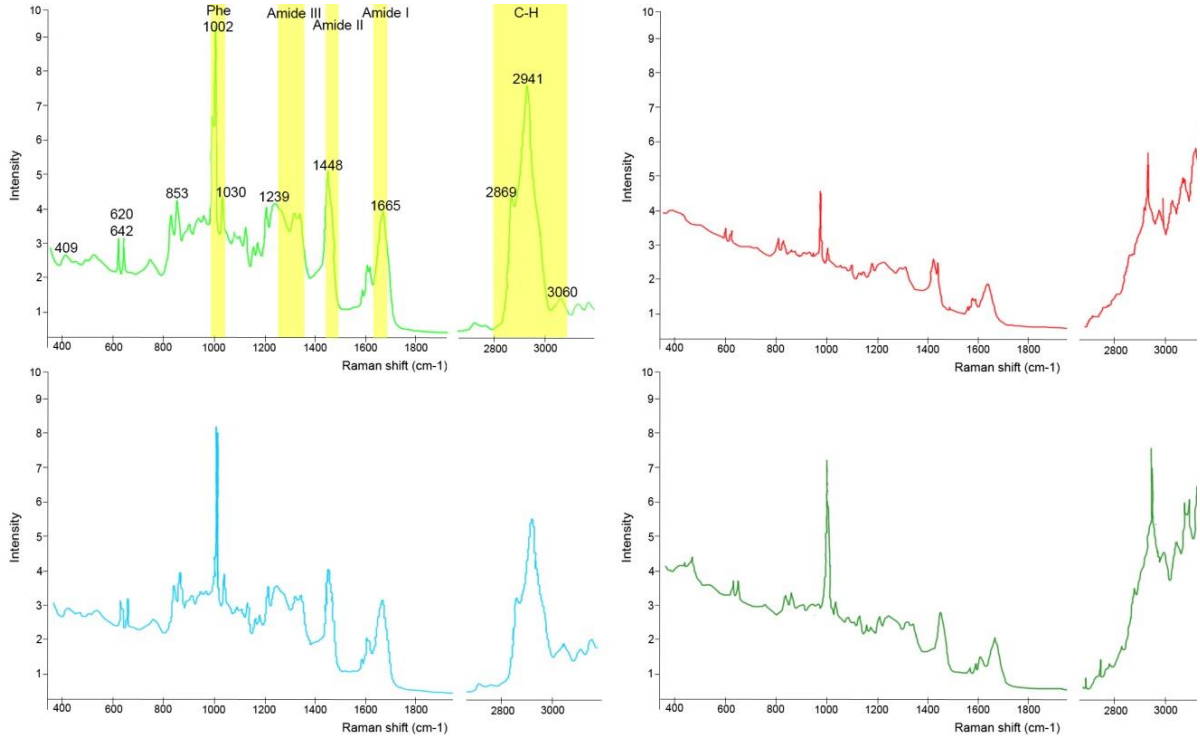

Figure 4.5: Raman spectra from adhesive samples of four different animals showing consistency in the range of peaks observed across samples.

The peaks of the Raman fingerprint region (400-1700 cm^{-1}) were assigned to amino acids according to the peak maximum values (Table 4.2). Where many amino acid Raman spectra contained the same peak, the amino acid composition of *L. anatifera* adhesive was also taken into consideration (Table 4.1). A selection of amino acid Raman spectra was superimposed on the *L. anatifera* adhesive Raman spectrum in order to visually demonstrate how a combination of amino acids could produce the *L. anatifera* adhesive Raman spectrum (Figure 4.6). Specific amino acids were selected based upon best fit to the *L. anatifera* adhesive spectrum or their prevalence in *L. anatifera* adhesive.

Bands consistent with tyrosine (Tyr) were observed in the barnacle adhesive spectra despite *L. anatifera* adhesive containing a relatively low amount of Tyr (3.69%, Walker & Youngson, 1975). A doublet at ~830/850 cm^{-1} that is considered to be the primary marker of Tyr; the stronger 853 cm^{-1} peak observed in the current results could indicate that the Tyr is 'exposed' and available to act as both a hydrogen donor and acceptor (Tu, 2003). However, some caution is required as two common amino acids in barnacle adhesive, alanine (Ala) and serine (Ser), are also characterised by strong bands at 853

cm^{-1}; these likely contributed to the strength of the 853 cm^{-1} band in the *L. anatifera* adhesive.

The strong Raman peaks that typically indicate cysteine (Cys) were not observed in *L. anatifera* adhesive. As a monomer, Cys produces two strong Raman peaks at approximately 660-685 cm^{-1} and 2500 cm^{-1}, assigned to C–S and S–H bonds, respectively (Edsall et al., 1950; Movasaghi et al., 2007). In contrast, the dimer cystine has a very strong peak at approximately 500-540 cm^{-1}, due to the disulfide bond (S–S) (Edsall et al., 1950; Movasaghi et al., 2007; Zhu et al., 2011). This strong Raman peak was also not present in *L. anatifera*; a weak, broad peak at 523 cm^{-1} may be due to disulfide bonds but could also have been caused by ring deformation of aromatic amino acids (Zhu et al., 2011).

Table 4.1: Adhesive amino acid compositions (residues per 1000) from *Lepas anatifera* (Walker & Youngson, 1975), *Dosima fascicularis* (Barnes & Blackstock, 1974), *Megabalanus rosa* (Kamino et al., 1996), *Balanus crenatus* and *Chirona hameri* (Walker, 1972). *Cystine/2 (dimeric state was measured); †Cysteic acid. Bold lettering indicates AA for which Raman peaks are presented visually in Figure 5.6.

AA	*L. anatifera*	*D. fascicularis*	*M. rosa*	*B. crenatus*	*C. hameri*
Leu	**97.9**	**97.6**	**82.8**	**81.0**	**87.8**
Gly	**95.7**	**87.1**	**79.2**	**85.9**	**82.7**
Glu	**91.0**	**114.1**	**91.5**	**86.3**	**90.5**
Ala	**89.9**	**98.8**	**74.7**	**64.7**	**68.7**
Asp	**87.5**	**101.2**	**90.7**	**82.8**	**78.9**
Ser	**87.5**	**98.8**	**99.1**	**76.9**	**113.6**
Val	66.5	77.6	72.5	21.9	27.4
Arg	60.7	61.2	56.0	61.3	58.5
Ile	**59.1**	**68.2**	**53.0**	**53.4**	**44.3**
Thr	52.5	63.5	70.5	62.3	65.6
Pro	**49.9**	**43.5**	**49.2**	**60.6**	**83.9**
Lys	38.5	24.7	56.7	67.9	54.7
Phe	**36.9**	**49.4**	**37.1**	**39.8**	**36.7**
Tyr	**36.9**	**1.2**	**41.8**	**53.8**	**49.2**
His	27.0	4.7	13.3	21.6	22.8
Cys	11.0*	2.4*	16.0†	72.8*	68.1*
Met	5.6	5.9	16.0	6.7	7.2

Table 4.2: Possible amino acid assignments of the peaks seen consistently in Raman spectra of barnacle adhesive. Amino acids (AA) were assigned based on similarity of peak maximum Raman shift (cm^{-1}) value (±5) and ordered according to most prevalent AA in *L. anatifera* adhesive. INT-intensity, br-broad, w-weak, m-moderate, o-p-out of plane, s-strong, sh-shoulder, vs-very strong.

Shift	INT	AA	Vibration assignment	Reference
409	w-br	Ala (m)		(De Gelder et al., 2007; Zhu et al., 2011)
523	w-br	Phe (w)	ring/C–C=O deformation	(De Gelder et al., 2007; Zhu et al., 2011)
		Cys (m)	S–S stretching	(Dollish et al., 1974; Movasaghi et al., 2007; Zhu et al., 2011)
620	w-m	Glu (m)		(Zhu et al., 2011)
		Phe (m)	C–C twisting	(De Gelder et al., 2007; Movasaghi et al., 2007; Zhu et al., 2011)
642	w-m	Pro (m)		(De Gelder et al., 2007; Zhu et al., 2011)
		Tyr (m)	C–C twisting	(De Gelder et al., 2007; Movasaghi et al., 2007; Zhu et al., 2011)
		Ala (w)		(De Gelder et al., 2007; Zhu et al., 2011)
750	w-br	Trp (s)	ring breathing	(De Gelder et al., 2007; Movasaghi et al., 2007; Zhu et al., 2011)
828	w-m	Ile (m)		(Zhu et al., 2011)
		Tyr (s)	ring breathing	(De Gelder et al., 2007; Movasaghi et al., 2007; Zhu et al., 2011)
853	w-m	Leu (m)	CH_3 rocking	(Filho et al., 2008)
		Ala(s)	C–N–C stretching	(De Gelder et al., 2007; Zhu et al., 2011)
		Ser (s)	C–C–O/C–C–N stretching	(De Gelder et al., 2007; Zhu et al., 2011)
		Val (s)		(De Gelder et al., 2007; Zhu et al., 2011)
		Ile (m)		(Zhu et al., 2011)
		Pro (sh)	ring breathing	(De Gelder et al., 2007; Movasaghi et al., 2007)
		Phe (m)		(De Gelder et al., 2007)
		Tyr (s)	ring breathing	(Dollish et al., 1974; Zhu et al., 2011)
899	w	Gly (s)	C–N–C stretching	(De Gelder et al., 2007; Zhu et al., 2011)
935	w	Asp (s)	O–H o-p-vibration	(Zhu et al., 2011)
		Thr (m)		(Zhu et al., 2011)
958	w	Met (w)		(Zhu et al., 2011)
		Pro (w)		(Zhu et al., 2011)
1002	s	Phe (s)	ring breathing	(De Gelder et al., 2007; Dollish et al., 1974; Zhu et al., 2011)
1030	w-m	Leu (w)	C–N/C–C stretching	(Filho et al., 2008)
		Ile (m)		(Zhu et al., 2011)
		Pro (w)	ring breathing	(De Gelder et al., 2007; Zhu et al., 2011)
		Phe (s)	ring breathing	(De Gelder et al., 2007; Zhu et al., 2011)
1124	w-m	Glu (w)		(De Gelder et al., 2007)
		Asp(w)	NH_3^+ wagging	(Zhu et al., 2011)
		Ser (m)		(Zhu et al., 2011)
		Val (m)	NH_3^+ rocking	(De Gelder et al., 2007; Zhu et al., 2011)
		Arg (m)		(De Gelder et al., 2007)
1154	w	Gly (w)		(De Gelder et al., 2007)
		Phe (m)	C–H/ring deformation	(De Gelder et al., 2007; Zhu et al., 2011)
		Tyr (w)		(Zhu et al., 2011)
1170	w	Ile (w)		(Zhu et al., 2011)
1204	w-m	Lys (m)		(Zhu et al., 2011)
1239	m	Leu (w)	CH_2 torsion	(Zhu et al., 2011)
		Ala (w)		(De Gelder et al., 2007; Zhu et al., 2011)
		Pro (m)	CH_2 wagging	(De Gelder et al., 2007; Movasaghi et al., 2007; Zhu et al., 2011)
1316	m	Leu (w)	C–H deformation	(Zhu et al., 2011)
		Glu (m)		(De Gelder et al., 2007; Zhu et al., 2011)
		His (s)	NH_3^+ rocking	(De Gelder et al., 2007; Zhu et al., 2011)
1338	w-m	Asp (s)	C–H bending	(Zhu et al., 2011)
		Trp (s)	C–H bending	(De Gelder et al., 2007; Dollish et al., 1974; Zhu et al., 2011)
1448	m	Lys (m)		(Filho et al., 2008)
		Phe (w)		(De Gelder et al., 2007; Zhu et al., 2011)
		Met (m)	CH_3 deformation	(Zhu et al., 2011)
1604	w	Ala (w)	C–O–O^- stretching	(Zhu et al., 2011)
		Phe (m)	C–C bending	(De Gelder et al., 2007; Movasaghi et al., 2007)
1613	w	Ser (w)		(De Gelder et al., 2007)
		Tyr (m)	C=C stretching	(Movasaghi et al., 2007; Zhu et al., 2011)
		Trp (w)	C=C stretching	(De Gelder et al., 2007; Movasaghi et al., 2007; Zhu et al., 2011)
1665	w-m			

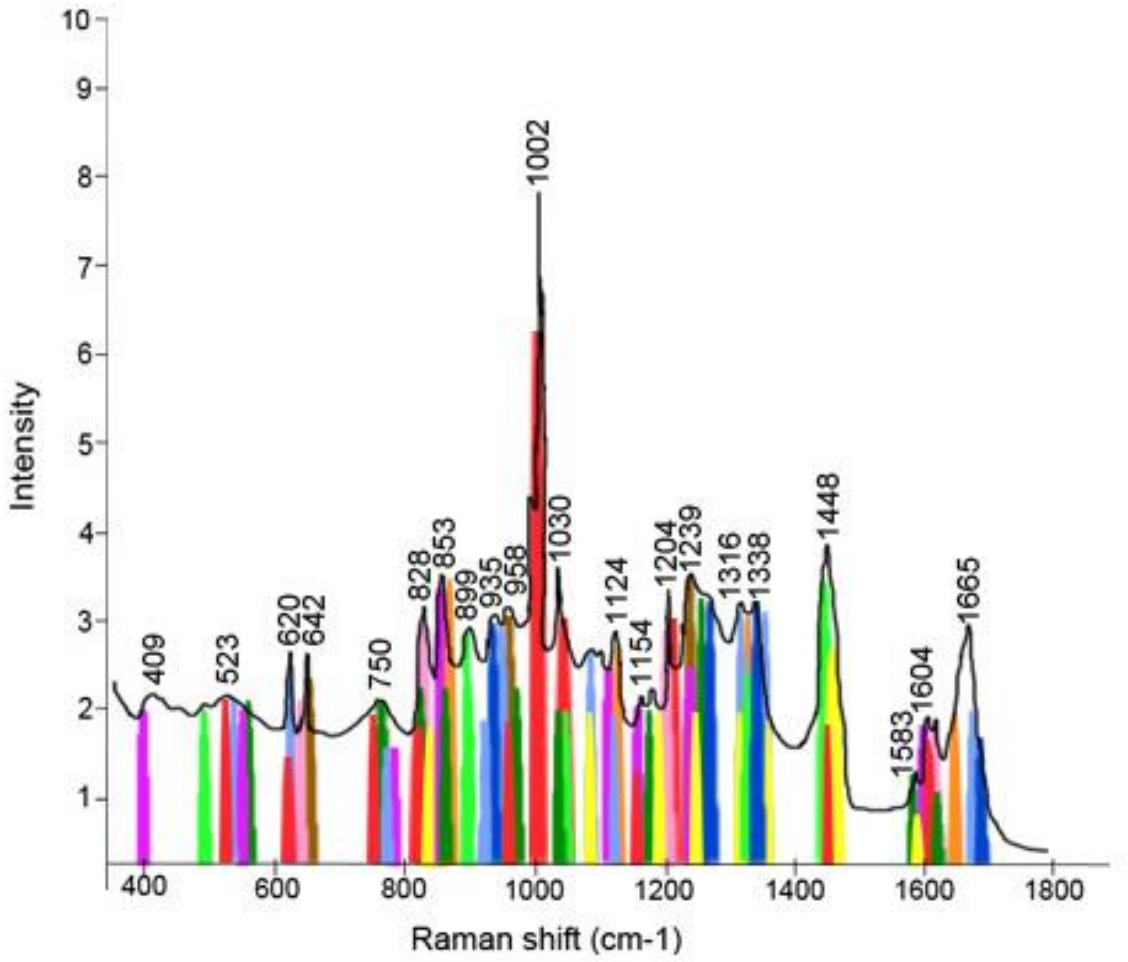

Figure 4.6: The Raman fingerprint region of the barnacle adhesive spectrum with amino acid Raman peaks superimposed beneath. Leu-yellow; Gly-light green; Glu-light blue; Ala-purple; Asp-dark blue; Ser-orange, Ile-dark green; Pro-brown; Phe-red; Tyr-pink.

The Raman spectrum from the adhesive of *L. anatifera* was compared with that of *B. crenatus* (Wiegemann et al., 2006); while there were some similarities, many of the peaks present in *L. anatifera* were absent in *B. crenatus* and vice versa (Table 4.3 & Figure 4.7)

Table 4.3: Suggested explanation of species-specific differences in Raman spectra of adhesive from *L.anatifera* (present study) and *B. crenatus* (Wiegemann et al., 2006). AA assignments based on species-specific differences in adhesive AA composition (reported previously by Walker and Youngson (1975) and Walker (1972)) and AA Raman spectra (De Gelder et al., 2007; Zhu et al., 2011).

L. anatifera	Assignment	*B. crenatus*	Assignment
642	Ala	770	Cys, Lys
853	Ala, Ser, Val	1015	Phe
1002	Phe	1280	Lys, Pro
1030	Leu, Ile	1390	Cys, Lys, Pro
1239	Leu, Ala	1475	Lys
1665	Amide I	1580	Phe

A distinctive feature of the *B. crenatus* adhesive Raman spectrum was the lack of the Tyr doublet, which was surprising as *B. crenatus* adhesive has a higher amount of Tyr (Walker, 1972) than *L. anatifera* (Walker & Youngson, 1975). The strong peaks at 770 cm^{-1} and 1390 cm^{-1} in the *B. crenatus* adhesive spectrum could be related to the high cystine content of *B. crenatus*, as strong peaks at 785 cm^{-1} and 1385 cm^{-1} are present in the cystine Raman spectrum (Zhu et al., 2011). Also, a peak at approximately 520 cm^{-1}, indicative of S–S bonds (Zhu et al., 2011), is stronger and more distinct in *B. crenatus* than in *L. anatifera*, however, C–S bonds (~670 cm^{-1}) and S–H bonds (~2500 cm^{-1}) were not present. *B. crenatus* adhesive also contains a high proportion of Lys (Walker, 1972), which may have contributed to the peaks at 770, 1280, 1390 and 1475 cm^{-1} (Zhu et al., 2011). Pro, which is relatively enriched in *B. crenatus* adhesive (Walker, 1972), also has moderately strong peaks close to 1280 and 1390 cm^{-1} (De Gelder et al., 2007). The *B. crenatus* adhesive Raman spectra was not dominated by Phe, despite the two species having similar Phe content, and also lacked any distinguishable Amide I peak, a general feature of proteins.

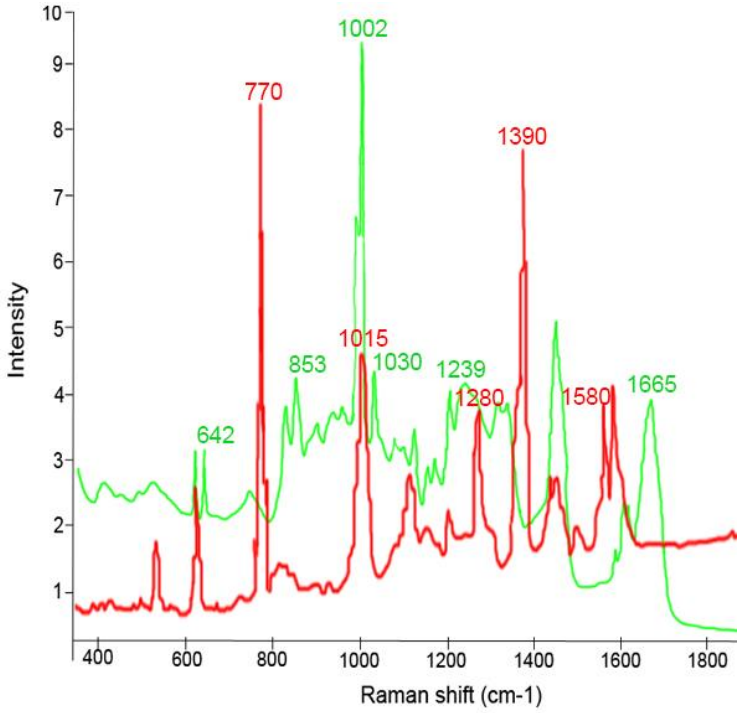

Figure 4.7: Raman spectra of the adhesive from *L. anatifera* (green) and *B. crenatus* (red), with prominent peaks that are unique to each species labelled.

4.3.6 FTIR spectroscopy

FTIR analyses of freeze-dried adhesive from four different specimens showed highly consistent spectra (only one is presented, for brevity: Figure 4.8). The strongest peaks, at 1620 and 1514, were assigned to Amide I (1600-1700) and Amide II (1500-1600), respectively (Movasaghi et al., 2008). A weaker peak at 1240 cm^{-1} corresponds to Amide III (1220-1350; Movasaghi et al., 2008). The strong, broad peak at 3220 indicates Amide A and B, which result from N–H stretching of the protein backbone.

FTIR spectral peaks from *L. anatifera* adhesive can be compared to results from *Amphibalanus amphitrite* (Barlow et al., 2009), *A. reticulatus* (Raman & Kumar, 2011; Sangeetha et al., 2010) and *A. improvisus* (Berglin & Gatenholm, 2003) (Table 4.4). Unlike in other species, the calcium carbonate ($CaCO_3$) signal was not present in *L. anatifera* adhesive, with no prominent peaks below 1000 cm^{-1}. Carbonate absorbs strongly at 1400 cm^{-1} (Berglin & Gatenholm, 2003); the present results show a weak shoulder to be present in this region, which was instead assigned to CH_3 deformation (Movasaghi et al., 2008). A broad region with at least three individual peaks between 1080 and 1160 cm^{-1} is likely to be associated with the C–OH bonds of oligosaccharide or polysaccharide carbohydrates (Movasaghi et al., 2008). There was no indication of phosphorylated proteins, which have a peak at 970 cm^{-1} (Movasaghi et al., 2008), in the FTIR spectrum of *L. anatifera* or any other barnacle species. Cysteine was also absent from the spectra, with no S–H peak at 2550 cm^{-1} (Barth, 2007) and no S–C peak between 680-820 cm^{-1} (Wolpert & Hellwig, 2006).

Table 4.4: FTIR peaks (cm^{-1}) for *L. anatifera* adhesive compared to *A. amphitrite* (Barlow et al., 2009), *A. reticulatus* (Raman & Kumar, 2011) and *A. improvisus* adhesive on PMMA (left column) and PDMS (right column) (Berglin & Gatenholm, 2003). Intensity: w-weak, m-moderate, s-strong, sh-shoulder. Peaks from spectra of dry or dry-pelleted adhesive, except *.

Assignment	*L. anatifera*	Intensity	*A. amphitrite*	*A. reticulatus*	*A. improvisus*	
$CaCO_3$				713	713	
$CaCO_3$			872	876	876	
C–O stretching (carbohydrates)	1080-1160	w	1081*	1055-1195	1090-1150	1120
Amide III	1240	m	1234			1250
Amide III			1313			
$CaCO_3$, CH_3 deformation	1400	w	1350-1420		1400	1390
CH_2/CH_3 deformation	1440	m	1425-1480	1428-1439		1450
Amide II	1514	s	1500-1580			1530
Amide I	1620	s	1600-1700	1625-1638		1650
C=O vibration				1793	1750	
S–H stretching				2516-2519		
C–H stretching	2820-2940	m	2800-3000	2835-3009		
Amide B	3200	sh	3060			
Amide A	3220	s	3280	3434		

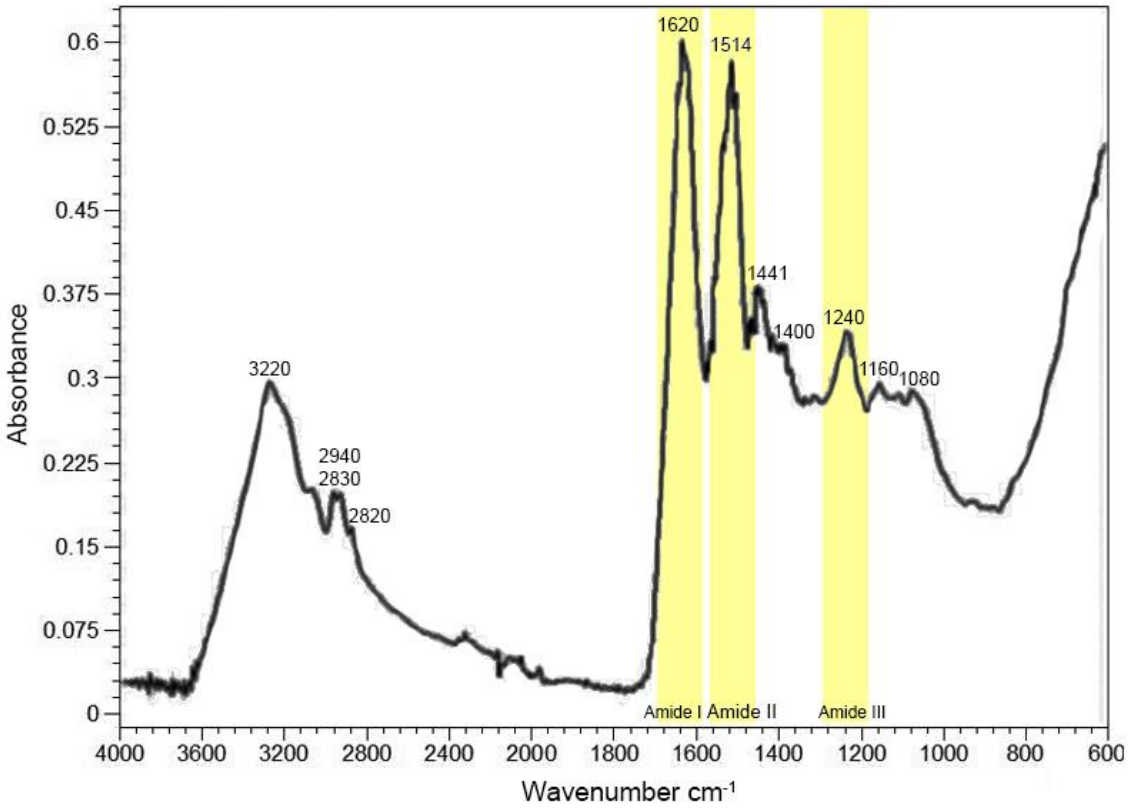

Figure 4.8: FTIR spectrum of barnacle adhesive, with prominent peaks labelled and the amide bands of proteins highlighted.

4.4 Discussion

The combination of chemical analyses used in the current study was chosen to provide complementary information to previous reports regarding the mechanisms involved in barnacle bioadhesion. A particular emphasis was placed on understanding the importance of chemical elements within the adhesive, as well as investigating covalent cross-linking by disulfide bonding or tyrosine associated chemistry, which have featured prominently in recent discussions (e.g., Burden et al., 2012; Kamino et al., 2012; Wiegemann et al., 2006). Species comparisons were considered in order to determine which, if any, mechanisms are ubiquitous to all barnacle adhesives. This is the first time energy dispersive spectrometry (EDS), Raman spectroscopy or Fourier transform infrared spectroscopy (FTIR) have been applied to *L. anatifera* and only the second such study to focus on a stalked barnacle species (*Dosima fascicularis* has recently been subject to examination by Zheden et al. (2013)).

We present the first reported elemental examination of the barnacle adhesive gland tissue and compare it to the elements observed in the adhesive. A clear increase was observed in the number of elements present in the plaque relative to the gland. This may be an indication that the glandular precursor to the cured adhesive is in a dilute form, with the concentration of some adhesive elements being too low to detect with EDS. This possibility may apply to sulfur, which was absent in the adhesive glands despite low levels of Cys and Met being reported in *L. anatifera* adhesive, yet always present in the adhesive. The differences between adhesive gland and cured adhesive chemistry could also be a reflection of a ‘maturing’ process in the adhesive, involving the addition of further functional elements from a different source. Alternatively, the differences could reflect chemical aspects of the general cellular environment and marine environment, which are unrelated to the adhesive curing. This may have been the reason for variable detection of Al, Si, Na, Cl, Mg, K and Fe. These elements may be incorporated into marine adhesives from seawater in a similar way to fish otoliths (e.g., Campana, 1999), thus elemental signatures may vary from sample to sample, according to the location that the barnacles had been sourced from.

Inside the adhesive gland a consistent EDS signal was only observed for the elements associated with the protein backbone (C, O and N), Na and Mg. In the tubeworm *P.*

californica distinctive granules containing Mg have been reported to be present in the adhesive gland system (Wang & Stewart, 2012), however the different structural components in the adhesive glands of *L. anatifera* were not observed have distinctive elemental profiles. A much wider range of elements was detectable in the adhesive plaque of *L. anatifera* compared with the gland tissue. The plaque composition was in agreement with reports from other barnacle species (Berglin & Gatenholm, 2003; Sullan et al., 2009; Walker, 1972), with a number of additional elements observed in *L. anatifera* adhesive. For example, Al and Si were present in *L. anatifera* adhesive, while in previous reports these elements were concluded to have originated from the aluminium foil and PDMS substrates used during analyses (Berglin & Gatenholm, 2003; Sullan et al., 2009). Al and Si may be an environmental addition to natural adhesives; the ions Cl^-, Na^+, SO_4^-, Mg^{++}, Ca^{++} and K^+ make up more than 99% of the salts in seawater (Pilson, 2013) and the same range of elements was observed in *L. anatifera* adhesive.

Interestingly, phosphorus was almost always absent in *L. anatifera* adhesive, in contrast to previous reports from other barnacle species (*B. crenatus* and *C. hameri* (Walker, 1972); *D. fascicularis* (Zheden et al., 2013)). The variable presence of P indicates an environmental contribution and that P is not an important functional element in barnacle adhesive. This was further supported by spectroscopy analyses ruling out any strong presence of phosphorylated proteins, in agreement with our previous immunohistochemical study showing no evidence of phosphorylated serines (Jonker et al., 2012). An absence of significant levels of phosphorylation is an important point of distinction, as this aspect of barnacle adhesion contradicts virtually all other wet-adhesion animal models, including the mussel, tubeworm, sea-cucumber, caddisfly larvae and kelp spores (Stewart et al., 2011). The transition metals (chiefly Fe) that also play functional roles in other underwater adhesives (mussels (Harrington et al., 2010) and gastropods (Werneke et al., 2007)) were not a large component of cured *L. anatifera* adhesive and no indication of protein-metal interactions was detected.

The presence of S and Ca in *L. anatifera* adhesive concurred with reports of other barnacle species (Berglin & Gatenholm, 2003; Zheden et al., 2013); however there was no evidence to suggest that either of these elements play functional roles. In the older literature, barnacle adhesive was proposed to be predominately $CaCO_3$ in a protein

matrix (Otness & Medcalf, 1972) and this was supported by Berglin and Gatenholm (2003), who concluded that $CaCO_3$ is incorporated into the adhesive matrix during adhesive plaque formation. However, observations regarding the $CaCO_3$ content of barnacle adhesive have in all cases been reported for species that possess a calcareous base plate which is adhered to the substratum. It is impossible using calcareous based species to distinguish whether $CaCO_3$ has been detected from the adhesive or the base-plate (Barlow et al., 2009; Raman & Kumar, 2011; Sangeetha et al., 2010). *L. anatifera* has a membranous stalk in place of a calcareous base-plate, thus if Ca is detected, its presence is independent of the base-plate. Ca was sometimes detected by SEM-EDS in *L. anatifera* adhesive, however FTIR analyses confirmed that the Ca content was not present in the form of $CaCO_3$; from this we can conclude that $CaCO_3$ is not a prerequisite of barnacle adhesion.

Regarding other possible roles for Ca found in *L. anatifera* adhesive, the Ca bridging that has been speculated to occur in other marine adhesives (Stewart & Wang, 2010) is associated with phosphorylation, which is not present in barnacle adhesive. Calcium could be incorporated into the adhesive in some other form for mechanical strength, such as Ca bridging with acidic residues, however because Ca was not consistently detected in every sample, it may not be functionally significant. The significance of lack of $CaCO_3$ involvement in *L. anatifera* adhesive pertains to a postulated link between barnacle adhesive secretion and barnacle growth (Fyhn & Costlow, 1976; Kamino, 2006). Recent evidence has pointed to the presence of two adhesive secretions being produced by barnacles, one of which is produced continuously as the barnacle grows and the other at intervals (Burden et al., 2012). The presence of $CaCO_3$ in barnacle adhesive would have provided a further link between shell growth and adhesive curing, however the results of the current study show that any link between barnacle adhesive secretion and barnacle growth clearly does not involve $CaCO_3$, although it remains possible that other elements of the exoskeleton, such as the cuticle, may be involved.

Sulfur was consistently present in cured adhesive samples from *L. anatifera*, though it could not be detected in the adhesive glands. Similar results were seen in the adhesive of *A. improvisus* (Berglin & Gatenholm, 2003) and *D. fascicularis* (Zheden et al., 2013). There has been much interest in what form S may take in barnacle adhesive; the

current spectroscopic results show that cysteine (S–H bonds) and cystine (S–S bonds) did not make any significant contribution to the sulfur content of *L. anatifera* adhesive. In agreement with this was the lack of a peak indicating S–H bonds (2550 cm^{-1}) in FTIR studies of *A. amphitrite* (Barlow et al., 2009) and *A. improvisus* (Berglin & Gatenholm, 2003) adhesive, although Raman and Kumar (2011) reported S–H bonds to be present in *A. improvisus* adhesive. Raman spectra from *L. anatifera* adhesive also indicate a lack of S–H and S–C bonds, although a weak, broad peak was present in the region of S–S bonds (500-540 cm^{-1}). A similar weak peak in the S–S region in *B. crenatus* adhesive was previously discounted (Wiegemann et al., 2006). As *L. anatifera* adhesive contains relatively little cystine (Walker, 1987) compared to that of *B. crenatus* (Walker, 1972), the results indicate a very low potential for disulfide bridges to have a major structural influence on adhesive cross-linking. Disulfide bonds in barnacle adhesive are more likely to impart shape upon individual proteins, as has already been shown for cp-52k (Kamino et al., 2012) and cp-20k (Suzuki et al., 2006), a feature that is conserved across acorn barnacle species for cp-20k (He et al., 2013).

What became apparent during the course of the present study was a pronounced contrast between the Raman spectra of *L. anatifera* and *B. crenatus* adhesive. This is only the third Raman analysis of barnacle adhesive, but the contrast between acorn and lepadiform barnacles is evident. *L. anatifera* and *B. crenatus* each possessed unique spectral peaks, in contrast to close similarity between *D. fascicularis* (Zheden et al., 2013) and *L. anatifera* spectra. The Tyr Raman doublet that was observed in *L. anatifera* adhesive was not present in *B. crenatus* adhesive, a finding that was at odds with the amino acid composition of each species' adhesives. While Raman has potential to become a highly informative analytical technique, interpreting the cause of contrasting Raman spectra remains speculative without more empirical studies of proteins using Raman. We attempted to circumvent this by mapping the Raman peaks for some amino acids (De Gelder et al., 2007; Zhu et al., 2011) that are common in the adhesive of *L. anatifera*, which could be of use in identifying the causes behind differences between spectra. However, the process also highlighted that most Raman peaks could be caused by many amino acids, making definitive bond assignments quite difficult.

While evidence thus far indicates a high level of disparity between Raman spectra of acorn and stalked barnacle adhesive, and further differences between adult barnacles and cyprid larvae, the latter of which is characterised by the strong presence of carotenoids (Schmidt et al., 2009), some features are consistent to all barnacle adhesive Raman investigations. No protein-metal interactions have been detected in barnacle adhesive, suggesting that these play no part in barnacle adhesion. Recent evidence suggests that non-DOPA phenolics may have some role in the adhesive, as evidence for phenolic ring structures (including Tyr) was seen in FTIR spectra of *A. amphitrite* adhesive (Barlow et al., 2009). The current study could neither confirm nor refute this hypothesis as, without D_2O treatment, the Amide II peak overlapped and obscured any possible contribution from phenolic rings. However, Raman provided evidence that Tyr is contributing to *L. anatifera* adhesive. The significance of Tyr in barnacle adhesion in general is somewhat difficult to reconcile with the species specific variation that has been reported for Tyr residues, including extremely low Tyr content in stalked barnacle species *D. fascicularis* (Barnes & Blackstock, 1974; Kamino et al., 1996; Walker, 1972; Walker & Youngson, 1975).

The presence of fibrillar cross-β-sheet structures has recently been suggested to be an important motif in barnacle adhesion (Barlow et al., 2010; Sun et al., 2007) and spectroscopic analytical techniques using FTIR, circular dichroism and Raman spectra can provide strong evidence regarding protein secondary structure. Secondary structure analysis can currently be performed through deconvolution and peak-fitting of FTIR spectra, however in order to avoid interference from H_2O the sample must be dissolved in D_2O (Byler & Susi, 1986; Lórenz-Fonfría & Padrós, 2004); unfortunately the insolubility of barnacle adhesive often prevents the application of common techniques, as in the current study. However, future Raman investigations of barnacle adhesive could also focus on secondary structure prediction. Raman spectra are not confounded by H_2O interference and methods are already being developed to robustly predict secondary structure using the relative intensities of the Amide I, Amide III and methylene deformation bands (Tu, 2003). More specific information regarding the mechanisms of barnacle adhesive could be gained through the use of surface-enhanced Raman spectroscopy (SERS) to investigate individual proteins absorbed to different substrates, for example, using the recombinant cp-19k and cp-20k proteins that have already been developed (Mori et al., 2007; Urushida et al., 2007). The use of SERS to

understand the surface chemistry properties of biological molecules has recently been reported to produce much narrower Amide I bands, providing the potential to increase the accuracy of protein secondary structure prediction (Gullekson et al., 2011). A combination of analyses such as these could help us to further understand the secondary structures that give shape and cohesion to the adhesive and the chemical features of adhesive protein adsorption to different substrates.

4.5 References

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5 The adhesive proteins of *Lepas anatifera*

5.1 Introduction

Adhesion to a substrate is a common phenomenon amongst marine organisms, who have adapted to the dense, gravity-defying medium that is seawater. Adhesion can be permanent or temporary, and is particularly developed in invertebrates (Walker, 1987). Underwater adhesives have long been known to be comprised of combinations of proteins and polysaccharides, or proteins incorporating polysaccharides (Young & Crisp, 1982). However, the well-studied permanent adhesives of mussels, tubeworms and barnacles are almost entirely comprised of protein (Jensen & Morse, 1988; Waite, 1983b; Walker, 1972). Representing three different phyla (Mollusca, Annelida and Arthropoda, respectively), each of these animals has developed a unique adhesive that consists of a complex of proteins that interact with each other and the substrate to form a strong, permanent bond.

The adhesives produced by members of the phyla Mollusca and Annelida share the functional mechanisms L-3,4-dihydroxyphenylalanine (DOPA) and phosphorylated serine (pSer) (Flammang et al., 2009; Stewart et al., 2004; Waite & Tanzer, 1981). Barnacle adhesive (Arthropoda) contains neither of these common modes of aquatic adhesion. Molluscs and annelids are more closely related than either of them are to the arthropods, with both being found within the clade Lophotrochozoa (Halanych, 2004), making their common utilisation of DOPA for adhesion somewhat less surprising. However, the second common mode of aquatic adhesion, pSer, is shared by molluscs, annelids and echinoderms (Flammang et al., 2009), the latter of which are in a completely different branch of the phylogenetic tree. While pSer is lacking in barnacles, it has been found in another arthropod adhesive; caddisfly larvae (Trichoptera, class Insecta) produce a silk-like adhesive that contains pSer, possibly as a specific adaptation for underwater adhesion (Stewart & Wang, 2010). This means that barnacles have clearly evolved a completely different system from both their near and far metazoan relatives.

Barnacle adhesive has been the subject of many investigations over the past decades, yet far less is known about these adhesive proteins than those of the mussel and tubeworm.

The presence of DOPA and pSer has quite clearly been ruled out (Jonker et al., 2012; Kamino et al., 1996), yet no functional motifs have been found in their place. The main molecular features of the proteins described thus far (from *Megabalanus rosa*) are the disproportionally high levels of the amino acids serine, threonine, glycine, alanine, valine and lysine in the small adhesive protein cp-19k (Urushida et al., 2007) and regular repetition of cysteine residues in the small adhesive protein cp-20k (Mori et al., 2007). The significant hydrophobicity of the two large proteins that make up the bulk of the adhesive (cp-100k and cp-52k) indicates the importance of hydrophobic interactions in this system (Kamino et al., 2012) and the predicted cross-β-sheet secondary structure of cp-100k may play an important role in cohesion (Kamino et al., 2000).

Unfortunately, our understanding of barnacle adhesion is further hindered by the lack of consistency in reports by different research groups. In *Perforatus perforatus* adhesive a prominent 39 kD protein band was unlike any other characterised barnacle adhesive proteins (Naldrett, 1993) while a prominent 36 kD band in *Amphibalanus eburneus* adhesive was different again (Naldrett & Kaplan, 1997). Despite the distinct protein sizes reported, some similarity between *A. eberneus* whole adhesive sequence fragments and cp-100k and cp-68k has been reported (Kamino et al., 2000). Proteins of a similar size range to that reported by Kamino and Shizuri (1998) have been found in *A. reticulatus* (Raman et al., 2013) and *A. improvisus* (Berglin & Gatenholm, 2003), however there have been no reports of attempts to isolate and characterise additional adhesive proteins (in contrast to searching for homologues of proteins already characterised by K. Kamino and colleagues).

It was the aim of this study to extend the base of knowledge regarding barnacle adhesive by investigating the proteins in the adhesive of the stalked barnacle *Lepas anatifera*, a species far removed from the acorn barnacles examined in the past. We aimed to solubilise the adhesive in order to compare the number and size of proteins to those in other barnacle species. Until recently (McEvilly, 2011; Zheden et al., 2013), stalked barnacle adhesive had not been biochemically analysed since 1976 (Barnes & Blackstock, 1976); we aimed to repeat and extend upon this, by examining the separated proteins using mass spectrometry and *de novo* sequencing. Finally, we aimed to isolate and amplify the cDNA encoding the separated adhesive proteins using PCR methods.

5.2 Methods

5.2.1 Collection of adhesive

The adhesive of *L. anatifera* was collected from samples taken from the wild, which had been adhered to various substrates. The adhesive plaque varied in appearance depending on the substrate, with the substrates most suitable for adhesive collection being glass and plastic, on account of the thick adhesive plaque that was produced. The adhesive extended over the base of the peduncle; it generally had a rubbery consistency and small pieces could easily be pulled away from the cuticle of the barnacle with sterile forceps. Great care was taken to not pierce the cuticle of the barnacle, and adhesive from any animal that was wounded was not used for later investigations. In addition, because the plaque was generally quite large and visible, sections of the adhesive were carefully shaved away with a clean razor blade and saved for protein analyses. The adhesive that was removed was examined under a stereomicroscope and any visible pieces of dirt, algae and other debris were removed.

The adhesive was also collected in its liquid form, before it hardened. This was done by carefully prising the adhesive plaque away from the cuticle of the peduncle, then inserting a fine pipette tip (0.2-2 µl pipettor) into the exposed gap. In some instances a small amount of liquid could be extracted in this way from between the base of the barnacle peduncle and the adhesive plaque. This was pipetted into a sterile 1.5 mL eppendorf, which was immediately frozen at -70 °C.

5.2.2 Solubilisation of adhesive

Adhesive samples were freeze-dried or desiccated within the tubes that they had been stored in and then kept in a desiccator at room temperature. Initial attempts to solubilise the hardened adhesive were made using a general buffer commonly used for cell lysis (7 M urea, 2 M thiourea, 1% DTT (w/v), 4% CHAPS (w/v)). The freeze-dried samples were pooled and weighed, then returned to the same tubes, to which 500 µL buffer was added (0.5-1.0 mg adhesive to 100-500 µL buffer). Best results were produced when the samples were combined with 500 µl of buffer, heated to 35 °C for one hour, with regular vortexing, then centrifuged for 10 minutes at 20,000 rpm. Following these steps, the clear supernatant was used for the separation. Various detergents that were used in

place of CHAPS gave the same results. Protein precipitation with ice-cold acetone was attempted (according to a general protocol) to produce clearer bands, however this resulted in a high loss of protein. Solubilisation was also attempted with a buffer of guanidine hydrochloride, according to the method of Kamino et al. (2000), modified to suit the equipment available. However this produced few protein bands and was not as successful or repeatable as the urea/thiourea buffer.

Freeze-dried liquid adhesive was only available in small quantities. Therefore this was treated with the urea/thiourea buffer, as it showed the most promising results.

5.2.3 One-dimensional SDS-PAGE

For 1D SDS-PAGE a 12% acrylamide separating gel was prepared (Table 5.1) and poured between the electrophoresis plates, then topped with a thin layer of butanol. The separating gel was left to set for at least one hour before the stacking gel was prepared.

Table 5.1: Composition of the separating gel for SDS-PAGE.

Separating gel	
Distilled H_2O	8.7 ml
1.5M Tris-HCl pH 8.8	5 ml
10% SDS	200 ul
40 w/v acrylamide bisacrylamide	6 ml
10% ammonium persulfate (APS)	100 ul
TEMED	20 ul

Before pouring the stacking gel (Table 5.2) the butanol was carefully removed and the separating gel flushed with water. The stacking gel was carefully poured on top of the separating gel and the comb inserted immediately. The gel was left to set for at least two hours prior to use.

Table 5.2: Composition of the stacking gel for SDS-PAGE

Stacking gel	
Distilled H_2O	6.43 ml
0.5M Tris-base (brought to pH 6.8 with HCl)	2.5 ml
10% SDS	100 ul
40% w/v acrylamide/bisacrylamide	1 ml
10% APS	50 ul
TEMED	10 ul

After the gels had been given sufficient time to fully set, 10 µl of each sample (clean supernatant only) was placed in a clean 0.5 ml tube with 10 µl of sample buffer (0.06 M Tris-base, 2% (w/v) SDS, 10% (v/v) glycerol, 0.004% (w/v) Bromophenol blue) and heated to 90 °C for 10 minutes. The gel was placed into a vertical electrophoresis rig with running buffer (Thermo Scientific). The samples were loaded into the gel alongside a broad range protein molecular weight ladder (Promega) and run at 150 V until the loading dye reached the bottom of the gel (approximately 30 minutes). Gels were washed in milliQ-H_2O for 5 minutes (repeated three times) and stained in Coomassie blue (Thermo Scientific) overnight, then de-stained in milliQ-H_2O until the washing water was clear. Gels were imaged with an Epson scanner and clear bands were removed with a clean razor blade and placed at -20 °C.

5.2.4 Mass spectrometry

Bands of 30, 70, 90, 110 and 130 kD from 1D SDS-PAGE were freeze-dried and sent to the Analytical Unit of the Instituto de Biologia Experimental e Tecnológica (IBET) & Instituto de Tecnologia Química e Biológica (ITQB-UNL) at the Universidade Nova de Lisboa. The samples were reconstituted, cleaned, trypsin digested and subjected to mass spectrometry (MS). The resulting spectra were compared to databases to search for homologues, none of which were found. The samples were then subjected to MS TOF/TOF. PEAKS software was used to produce *de novo* amino acid sequences (Table 5.8).

5.2.5 Degenerate primer design

The length of the amino acid sequences produced by MS techniques ranged from 7 to 16 residues and therefore three different approaches were taken for primer design using these peptides (Figure 5.1). Short primers of 14 nucleotides were designed to flank the longest AA sequences (more than 14 AA) in order to amplify the short fragment sequence using normal PCR methods. Further primers were created from the shorter *de novo* sequences and were combined randomly. Primers for rapid amplification of cDNA ends (RACE) PCR were initially designed to be at least 23 nucleotides long and have a degeneracy of around 500 or below (Figure 5.1), though later primers were shorter, with higher degeneracy, out of necessity. Primers (produced by Biomers) were rehydrated and diluted according to manufacturer's protocol.

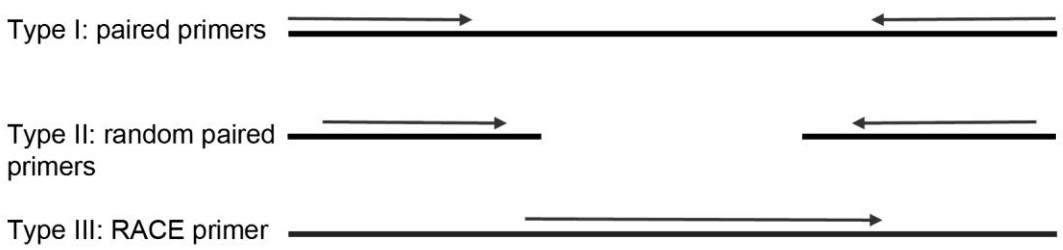

Figure 5.1: Schematic illustrating the design of primers from *de novo* sequences. Black lines indicate *de novo* sequences, arrows indicate primer location.

Table 5.3: List of primers designed for the isolation and amplification of cDNA fragments matching the *de novo* amino acid sequence fragments found through mass spectrometry. Primer names indicate primer type: P-paired, M-mixed/random paired, R-RACE. Brackets indicate undetermined residue.

***De-novo* sequence**	**Name**	**Band**	**Forward**	**Reverse**
YPREAATAAVSGPR	P01	110	tayccnmgngargc	nckngggnccnswna
RMPCAATAAVSGPR	P02	110	mgnatgccntgygc	
FEDFLVSNVQSFSR	P03	90	ttygargayttyyt	ncknswraanswyt
GSGATPYSRGGDGR	P04	70	ggnwsnggngcnac	nckngccrtcnccnc
YPGL(Q/K)PSTAANLLR	P05	70	tayccnggnytnma	ncknarnarrttng
	M01			ytknarnccnggrta
VVLVAKSHNSLYVEGR	M02	70	gtngtnytngtngc	ngcnacnarnacnac
	M03			nckngccytcnacrta
QQLALPSFVSQFR	M04	30	carcarytngcnyt	
F(L/V)(N/K)YFL(N/D)SR	M05	30	aantayttyytnra	tynarraartantt
LNNGLNVLAPR	M06	70, 90, 110	aayggnytnaaygt	acrttnarnccrtt
DMHPFFNPSR	M07	110	gayatgcayccnhk	mdnggrtgcatrtc
YSPMFSR	R01	30,90,110	taywsnccnatgttywsnmgn	ncknswraacatnggnswrta
GFSRSSNLVR	R02	30	ggnttywsnmgnwsnwsnaayyt	arrttnswnswncknswraancc
NYMLFTTR	R03	70,90,110	aaytayatgytnwtbacnacnmg	ckngtngtvawnarcatrtartt
MPALLVR	R04	90,110	atgccngcnytnytngtnmgn	ncknacnarnarngcnggcat
NPNGPFFYYK	R05	130	aayggnccnttyttytaytayaa	ttrtartaraaraanggnccrtt
GSGATPYSRAGGDGR	R06	70	ccntaywsnmgnggnggngaygg	ccrtcnccnccncknswrtangg
QGSSRFNISKNR	R07	110	mgnttyaayathwsnaaraaymg	ckrttyttnswdatrttraanck
RNTMCLFQAPR	R08	130	aayacnatgtgyytnttycargc	gcytgraanarrcacatngtrtt
DPMPLPVPSLLPR	R09	70	gayccnatgccnytnccngtncc	ggnacnggnarnggcatnggrtc
SSSTNGYFGMACNK	R10	30	tayttyggnatggcntgyaayaa	ttrttrcangccatnccraarta
HQPGNYFGMEMR	R11	30	ggnaaytayttyggnatggaratg	catytccatnccraartarttncc
RCCGNYQAYFNK	R12	90	aaytaycargcntayttyaayaa	ttrttraartangcytgrtartt
LMYGPQHDQPAAR	R13	130	atgtayggnccncarcaygayca	tgrtcrtgytgnggnccrtacat
LMYGPQHDQPAAR	R14	130	taygnccncarcaygaycarcc	ggytgrtcrtgytgnggnccrta
RYRNPQR	R15	30, 70, 110	mgntaymgnaayccncarmgn	nckytgnggrttnckrtanck

5.2.6 RNA extraction, cDNA synthesis and PCR

The entire stalk of small barnacles was removed, sectioned into small pieces and placed in sterile ependorfs containing RNAlater (Qiagen). The tissue was stored at 4 °C overnight, then moved to -70 °C. RNA was extracted using the RNeasy kit with Qiashredder columns (Qiagen) according to manufacturer's instructions for purification of total RNA from animal tissues. RNA quality was tested on a 1.5% agarose formaldehyde gel and a NanoDrop spectrophotometer was used to check for both purity

and concentration. RiboLock RNase inhibitor (Thermo Scientific) was added to the RNA solution prior to storage at -70 °C.

Extracted RNA was used to synthesise cDNA with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Concentration of cDNA was determined using a NanoDrop and the cDNA was diluted to 0.25 µg/µl with reagent grade H_2O (Thermo Scientific) before being stored at -20 °C.

PCR reactions were conducted with GoTaq (Promega) according to Table 5.4 and Table 5.5, in a gradient capable thermocycler. PCR reactions were run on a 1% agarose gel containing SybrSafe (Invitrogen) at 150 V for 25 minutes and immediately viewed and imaged. Gel bands of the desired size were carefully removed with a clean scalpel and placed in clean 1.5 ml eppendorf tubes, after which they were either stored at -20 °C or immediately purified.

Table 5.4: Components of the PCR reaction as used for all PCR reactions in this study, unless otherwise stated.

Reagent	Concentration	Volume (µl)
GoTaq flexi buffer	5X	10
DNTPs	10 mM	1
MgCl	25 mM	5
GoTaq	5 units/µl	0.25
cDNA	0.25 µg/µl	2.5
Sense primer	10 µM	2
Antisense primer	10 µM	2
H_2O		27.25

Table 5.5: Protocol for PCR reaction.

	Step	Temperature (°C)	Time
	Initial denature	94	2 minutes
35 cycles	Denature	94	30 seconds
	Annealing	48-62	30 seconds
	Extension	72	1 minute
	Final extension	72	5 minutes

5.2.7 RACE PCR

A modified version of the SMARTER II RACE kit (Clontech) was used to create cDNA for 3'-RACE and 5'-RACE PCR according to the manufacturer's protocol, using Superscript III reverse transcriptase (Invitrogen). PCR was then conducted using either GoTaq, according to the above protocol, with extension time increased to 3 minutes and final extension increased to 10 minutes, or PlatinumTaq (Invitrogen), a hot-start polymerase (Table 5.6 and Table 5.7). The PCR reaction was separated by gel electrophoresis, imaged and extracted as described above.

Table 5.6: Components of PCR reaction for rapid amplification of cDNA ends.

Reagent	Concentration	Volume (µl)
PCR buffer	10X	5
DNTPs	10 mM	1
$MgCl_2$	50 mM	1.5
PlatinumTaq	5 units/µl	0.2
cDNA	0.25 µg/µl	2.5
Sense primer	10 µm	1
Antisense primer	10 µm	1
H_2O		37.8

Table 5.7: Protocol for PCR reaction for rapid amplification of cDNA ends.

	Step	Temperature (°C)	Time
	Initial denature	94	4 minutes
35 cycles	Denature	94	30 seconds
	Annealing	48-62	30 seconds
	Extension	72	3 minute
	Final extension	72	10 minutes

5.2.8 Ligation, transformation and cloning

The PCR amplified products were purified from gel bands using the Qiaquick gel clean-up kit (Qiagen) according to the manufacturer's protocol, using reagent grade water (Thermo Scientific) to elute the DNA from the spin column in the final step. The purified product was either stored at -20 °C or immediately ligated and transformed.

The vector pCR-II-TOPO (Invitrogen) and TOP10 competent *E. coli* cells were used for all experiments, according to the manufacturer's protocol. Transformed cells were divided and spread onto two pre-warmed agar plates and incubated overnight at 37 °C.

Blue and white screening was used to identify colonies containing a vector insert. For each amplified product 10 white clones were picked from the plates and grown in a shaking incubator for 8 hours in 1 ml of LB broth at 37 °C, 200 rpm. Clones were screened via PCR using M13 sense and antisense primers, according to the protocols outlined in Table 5.4 and Table 5.5. Clones that contained inserts of the correct size were grown for a further 8 hours in 6 ml LB broth at 37 °C, 200 rpm. Plasmids were extracted using the PureYield plasmid miniprep system (Promega) according to the manufacturer's protocol. The concentration of the purified plasmid was measured on a NanoDrop and the plasmid inserts were subsequently sequenced using either the T7 or SP6 primers by GATC-biotech.

5.2.9 Sequence analysis

DNA sequences were analysed through a combination of Blast (NCBI), ORF-finder (NCBI), Translate-tool (ExPasy) and manual alignment with the programmes Mega-5 and GeneDoc.

5.3 Results

5.3.1 Solubilisation and separation

The hardened adhesive was almost completely solubilised using a buffer of urea and thiourea with DTT as a reductant. While a small portion of the adhesive remained solid, it was possible to separate the supernatant into at least 12 protein bands using 1D SDS-PAGE. Various methods were used to produce a clean separation of the proteins, with the most successful results being achieved through increasing the amount of urea/thiourea buffer used and centrifuging the sample to separate the solid from the dissolved substances. Biological replicates were run with samples collected from different locations at different points in time and the range of bands observed was the same. Intense bands were present at approximately 30, 70, 80, 90 and 110 kD (Figure 5.2, strong band at 70 kD observed in further repeats not shown here). A triplet of bands was present at approximately 60-68 kD.

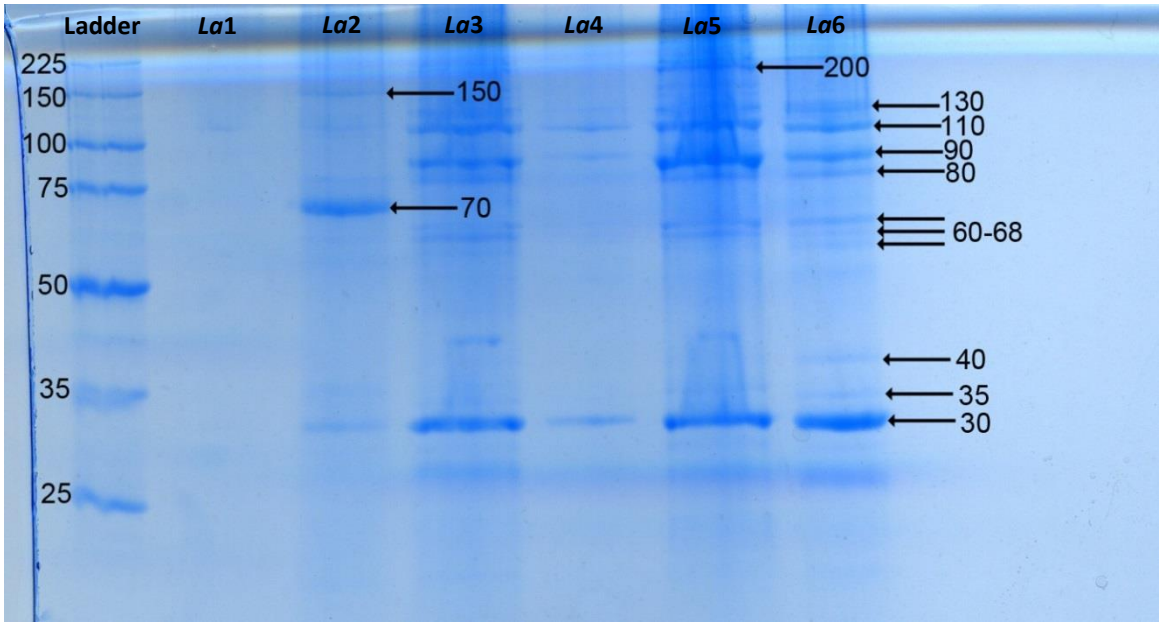

Figure 5.2: SDS-PAGE showing biological replicates of *L. anatifera* adhesive protein separation; left hand column is ladder of protein sizes, lane *La*1 contains acetone-precipitation sample and all other lanes contain urea/thiourea samples. Prominent, repeated bands were seen at approximately 30 kD, 90 kD and 110 kD. Fainter bands were seen at 35 kD, 40 kD, 60-68 kD, 70 kD, 80 kD, 130 kD, 150 kD and 200 kD.

The liquid adhesive had very low viscosity and, when pipetted onto a glass slide, generally left the pipette tip as bubbles, which retained their shape as they dried (Figure 5.3). The liquid dried in air over the course of several hours, into a thin, opaque film. Attempts to separate the proteins in the liquid adhesive using 1D SDS-PAGE were

unsuccessful; a pale smear was observed on the gel with no distinguishable protein bands.

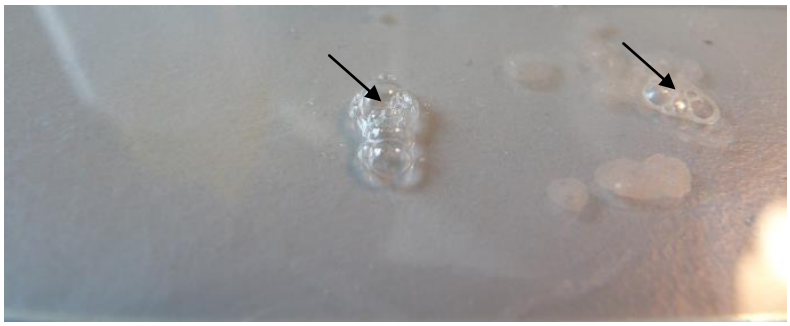

Figure 5.3: Liquid adhesive that was taken from beneath the hard adhesive of a specimen of *L. anatifera.*

5.3.2 Analysis of protein bands with mass spectrometry

Protein bands were extracted from the SDS gels and subjected to mass spectrometry. The spectra produced for each protein band had no database matches thus further analysis was conducted using *de novo* sequencing. The trypsin digests of each band were analysed twice by the IBET/ITQB-UNL, producing short sequences ranging in molecular weight from 815 to 1770 daltons (7-16 AA in length) (Table 5.8). Several of the sequences found were repeated across several of the bands that were analysed.

Table 5.8: *De novo* sequences from *L. anatifera* adhesive protein bands (listed along top row), produced by MS TOF/TOF and analysed by PEAKS. Molecular weights of each fragment are listed in the first column. Sequences found in more than one protein band are indicated in italics.

MW	30	70	90	110	130
815		MPGLLLR	*MPALLVR*	*MPALLVR*	
855		MSLMMSR			
870	WLVSLPR	VATLSHSR	LTPSSLPR		
903	*YSPMFSR*	*YSPFMSR*	*YSPMFSR*	*YSPMFSR*	FCGTCCNR
909	*MPAKPLPR*	*MPAKPLPR*	*MPAKPLPR*	*MPAKPLPR* LPGAWIPR	ASGTAHLPR
990	VGADSSGNGAR	RYRNPQR	RWSSNGKR	SASPERTSR	
1055		*YLSSLLFGR*	*YLSSLLFGR*	*YLSSLLFGR*	
1057	CNPLSLPGTR				
1061		*NYMLFTTR*	*NYMLFTTR*	*NYMLFTTR*	AQHSVLFCR
1065	SSLGLAGCCR				
1106	SNLYLQNVR SNLSNYGPVR			SARYLGANVR	
1122	GHGSALNLVR GFSRSSNLVR				
1147	YYSFPSDLR				
1158	QMVFYIDSR			QMVFYIDSR	
1174	FLNYFLNSR FVKYFLDSR				
1181		LDNGLNVHSGR VENLVGGLKPR	NNNLVGGLKPR	LNNGLNVLAPR	
1247				DMHPFFNPSR DMHPRHGNQR	NPNGPFFYYK
1250	YSGHLGFLNSR				
1337		GSGATPYSRGGDGR	SNCSQGSGLACAPR	SNRYSGGQTNPR	
1371	ANFSPLVSSFFR				
1393			RCCSNVLFAQPR	QGSSRFNISKNR	RNTMCLFQAPR
1431		DPMPLPVPSLLPR			
1443			FSLFNVPTLYSR	HPLNFVTPLYSR	
1444		DGSREAAYLPLPR RMKEAAYLPLPR			
1445				YPREAATAAVSGPR	LGYRRNLPLPR
1459		YPGLEAATAAQLVR			
1466	*FSQPYFYVPYR*		RCCGNYQAYFNK	*FSQPYFYVPYR*	SSSTGNYAQYMNK
1500		YPGLKPSTAANLLR YPGLQPSTAANLLR			LMYGPQHDQPAAR
1520	QQLALPSFVSQFR				
1530		NGPYQQNLQRRR	GPNYELQLQPNTR	RMPCAATAAVSGPR	SAQCNLQLQRRR
1674			FEDFLVSNVQSFSR		
1770			VVLVAKSHNSLYVEGR		

Some *de novo* sequences had 100% coverage matches to a single entry in protein databases (NCBI and uni-prot), however these matches were not significant; no two sequences from the same sample matched the same database entry (Table 5.9). This was to be expected, as it is known that barnacle adhesive proteins are unlike any known proteins. The longest *de novo* sequences (more than 11 AA) were subject to multiple BLAST analyses and compared to translated cDNA open reading frame sequences from a transcriptome of *Amphibalanus amphitrite* (Chen et al., 2011) and an unpublished database for *Tetraclita*, but these returned no matches.

Table 5.9: Comparison of MS spectra of *L. anatifera* adhesive protein band tryptic digest to the NCBI and uniprot protein databases.

***De-novo* sequence**	**Protein**	**Source**
MPAKPLPR	3-methyladenine DNA glycosylase	Bacteria, Xanthomonas
GFSRSSNLVR	Zinc-finger protein	Many
QMVFYIDSR	Predicted, uncharacterised protein	Sea urchin
DPMPLPVPSLLPR	Splicing factor	Many
GSGATPYSRGGDGR	Hypothetical protein	Bacteria, Pseudoalteromonas
SASPERTSR	Zinc finger protein	Mouse
LPGAWIPR	Uncharacterised/multiple	Sea urchin/others
QGSSRFNISKNR	Predicted serine/threonine phosphatase subunit	Sea urchin

5.3.3 Similarities between *L. anatifera* and *D. fascicularis* adhesive proteins

The results of MS *de novo* analyses were compared to two collections of *de novo* sequence fragments from the adhesive proteins of the stalked barnacle *Dosima fascicularis* (McEvilly, 2011; Zheden et al., 2013). Both studies agree that there are 10 to 11 proteins in the adhesive of *D. fascicularis*, however each study reported a different size range; 14 to 245 kD (McEvilly, 2011) and 47-205 kD (Zheden et al., 2013). The most consistent similarities between stalked barnacle adhesive protein separations were the presence of bands at approximately 70, 90, 110 and 150 kD (Table 5.10).

Table 5.10: Proteins observed in the adhesive of *L. anatifera* (current study) and *D. fascicularis*; 1: Zheden et al. (2013), 2: McEvilly (2011) partially polymerised adhesive, 3: McEvilly (2011) unpolymerised adhesive. All values in kD , bands that are consistent between species are indicated in bold, bands that were consistent between repeated 1D SDS-PAGE analyses for *L. anatifera* adhesive are indicted with *, bands that were weak or not always present in *L. anatifera* adhesive are indicated with w.

L. anatifera	*D. fascicularis*		
	1	**2**	**3**
26w		18	24
30*		30	
35w			
40w	47		
60-68w	63		
70*	**68**	**70**	**70**
80w			75
90*	**85**	**85**	**90**
110*	**111**	**110**	
130w*		140	140
		145	
150w	**149**	**150**	**155**
200w	205	200	

The majority of sequence fragments from the two *D. fascicularis* studies were not similar to each other, or to the *L. anatifera de novo* sequence fragments. The *L. anatifera* sequence MPALLVR, found in the 90 and 110 kD protein bands, was present as YPALLVR in a 70 kD protein band from *D. fascicularis* adhesive (McEvilly, 2011). Similarity was also seen between the fragments WLVSLPR (*L. anatifera* 30 kD) and AATVSLPR (*D. fascicularis* 14 kD (McEvilly, 2011)), CNPLSLPGTR (*L. anatifera* 30 kD) and TPLSLESVTR (*D. fascicularis* 60 kD (Zheden et al., 2013)), and FFEDFLVSNVQSFSR (*L. anatifera* 90 kD) and FEDFLVNNLNAFSR (*D. fascicularis* 63 kD (Zheden et al., 2013)).

5.3.4 Amplification of *de novo* fragments, including RACE PCR

Various primer pairs were designed as flanking primers to attempt to amplify the longest *de novo* sequences (see Table 5.3, 'P' primers), however it was not possible to amplify any product. These primers plus further primers were then used in random combinations (see Table 5.3, 'M' primers) to attempt to amplify sections of the isolated proteins, under the assumption that all the *de novo* sequences produced are a part of the same protein. Again, it was not possible to amplify any products.

RACE PCR was conducted using primers generated from *de novo* amino acid sequences. The majority of these PCRs resulted in no amplified products. However, some faint bands were seen of various sizes using 5' RACE. It was not known whether the bands seen in the SDS-PAGE gel represented a single protein, nor was it known where in that possible protein the *de novo* sequences exist; thus it was impossible to know by size alone whether the bands that appeared after 5'RACE were the correct product. Amplified products were subsequently extracted and cloned, however cloning was unsuccessful for the majority of PCR products. Successfully cloned products were sequenced, with ambiguous and primarily negative results returned.

A single primer based on the 70 kD band, R09, amplified various products, producing slightly smeared lanes with bands of approximately 400, 600 and 1500 bp and possibly further faint bands. The two clearest bands, at 600 and 1500 bp, were cloned and sequenced but multiple different sequences were returned, most of which did not contain the gene specific primer, any open reading frames (the translated sequences contained many stop codons) or significant similarity to any entries in the NCBI database. A single sequence from the 600 base pair band showed partial matches to GIDb and a tRNA uridine enzyme (about 40% coverage for each) (Table 5.11), however the sequence lacked the gene specific primer and any long open reading frame.

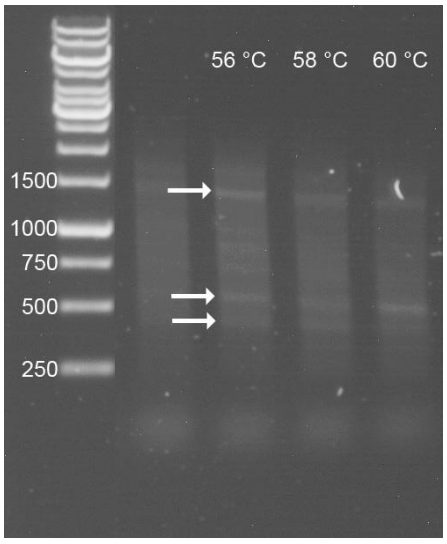

Figure 5.4: 5' RACE PCR with 70 kD band *de novo* primer R09 showing a smearing of faint products at a range of annealing temperatures.

The R12 primer, based on the 90 kD band, also amplified a product, however repetitions of the PCR experiment showed that the result was not repeatable. In addition, the bands produced were quite faint. A single product from these PCR experiments was cloned and sequenced (Table 5.11). The sequence returned was 966 base pairs in length and the R12 primer sequence was not present. The sequence did not contain any long open reading frames. BlastN found the sequence to partially match the mRNA sequence for Ultrabithorax Antennapedia, a homeobox gene, from a species of crayfish.

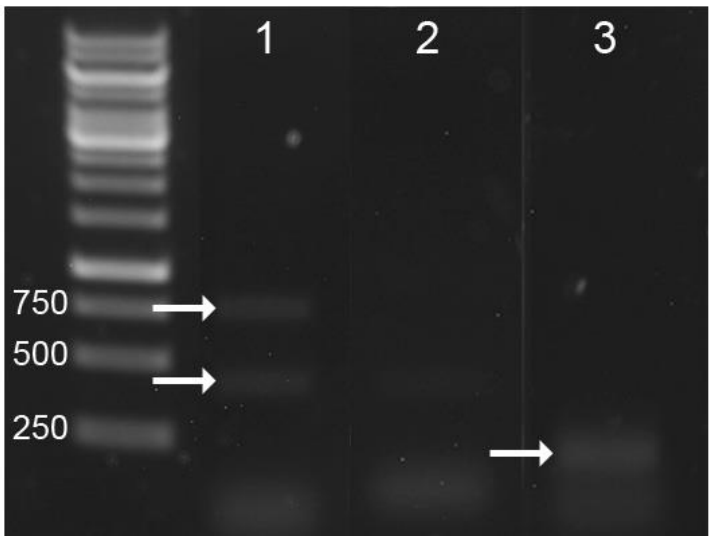

Figure 5.5: Three repetitions of 5' RACE PCR using 90 kD band *de novo* primer R12. PCR1 produced two products at approximately 400 and 750 bp, PCR2 produced a single faint product at approximately 400 bp and PCR3 produced a single faint product at approximately 250 bp. The 750 bp product from PCR1 was sequenced (Table 5.11)

Three 5' RACE primers based on *de novo* sequences from the 130 kD protein band produced amplified products, however the results were not repeatable in successive PCR experiments, varied with different annealing temperatures and were only faintly visible using agarose gel electrophoresis (Figure 5.6). The exception to this was PCR with primer R13, which in two repeats out of three produced an amplified product of 500 bp at all annealing temperatures. However, cloning and sequencing was only partially successful and the only sequence returned that showed any similarity to the NCBI database was from the R08 800 bp band, which despite having no long open reading frame was similar to both GIDb and a tRNA uridine enzyme (about 40% coverage each) (Table 5.11). This sequence was also exactly the same as that returned for the product of R09, based on the 70 kD band. No sequences returned for the bands shown in Figure 5.6 contained the gene specific primer sequences.

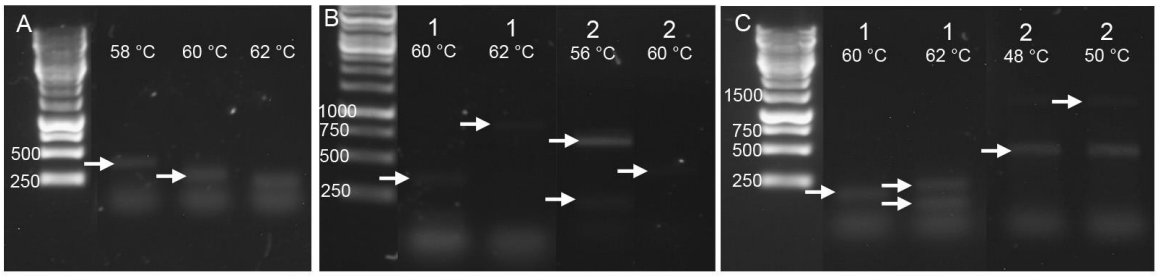

Figure 5.6: 5' RACE PCR using primers based on 130 kD band *de novo* sequences: A) primer R05 produced two different products at different annealing temperatures, approximately 350 bp at 58 °C and 250 bp at 60 °C; B) two PCR reactions using primer R08 with different results, products of approximately 350 and 800 bp were amplified in PCR1 and products of approximately 700, 350 and 250 bp were amplified in PCR2; C) two PCR reactions using primer R13 with different results, PCR1 produced bands at approximately 150, 200 and 250 bp while PCR2 produced bands at approximately 1500 and 500 bp.

De novo sequencing of the MS spectra produced some sequences that appeared in more than one of the protein band samples. RACE primers based on these sequences were created and almost all produced a similar smear when run through an agarose gel, with faint bands apparent at approximately 400, 600 and 1500 base pairs; the same pattern as was seen with R09, based on a sequence from the 70 kD band. After cloning and sequencing, most of the amplified products were found to have no highly similar matches in the NCBI database. Figure 5.7 shows the results from the three primers that amplified products with the most significant matches to entries in the NCBI database.

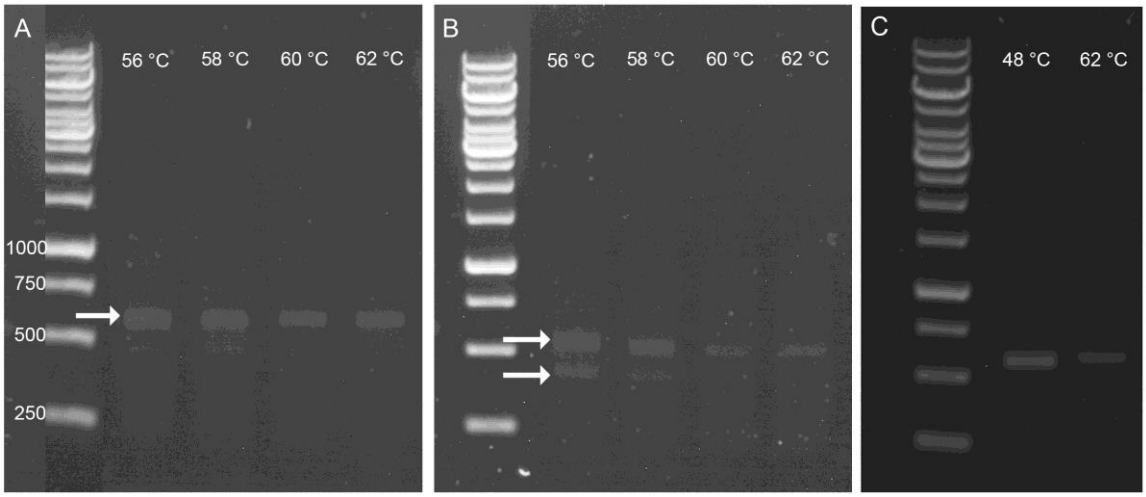

Figure 5.7: 5' RACE PCR results from the primers R01 (A), R03 (B) and R04 (C), all showing similar results with smearing apparent in A and B and an obvious band at approximately 600 bp in A, B and C

R01 and R03 produced very similar patterns on an agarose gel, while R04 produced a clearer gel with no smearing and a single band visible. The clearest products were cloned and sequenced; the 600 bp band from R01, the 400 and 600 bp bands from R04

and the 600 bp band from R04. The 600 bp products from R01 and R04 were exactly the same as each other and the products of R09 (based on the 70 kD protein band) and R08 (based on the 130 kD protein band) (Table 5.11). The 600 bp product of R04 was different and was found to be similar to Titin, a protein of the immunoglobin superfamily (Table 5.11). However, as with all other sequences derived from RACE PCR in this study the sequences had no long open reading frames and did not contain the gene specific primers used in the PCR reactions.

Table 5.11: Blast results for sequenced products of 5'RACE PCR reactions using the degenerate gene specific primers listed.

Primer	5'RACE BLAST result	Source	Coverage/Identity
R12	homeobox gene antennapedia	*Procambarus clarkia*	48%/81%
R01 R04 R08 R09	Glucose inhibited division protein B and tRNA uridine 5-carboxymethylaminomethyl modification enzyme	Many, bacterial	48%/56% 41%/63%
R03	Immunoglobin superfamily, Titin	Many, Insecta	97%/61%

5.4 Discussion

Protein characterisation results are heavily influenced by the methodologies used and discussion of the current results begins on this theme. Solubilisation of the freeze-dried adhesive was straightforward, in contrast to previous studies (Kamino et al., 2000; Kamino et al., 1996; Naldrett, 1993; Naldrett & Kaplan, 1997), however the adhesive was never fully solubilised and the results of protein analysis must be viewed in this light. The urea/thiourea/detergent protocols gave optimal solubilisation in the current study; this almost completely dissolved the adhesive samples, however in common with virtually all barnacle adhesive protein studies to date, incomplete solubilisation of adhesive remains a consistent feature (Kamino et al., 2000; Naldrett & Kaplan, 1997).

Separation and visualisation of the *L. anatifera* proteins in the soluble fraction of the hard adhesive showed intense protein bands at approximately 30, 70, 90, 110 and 130 kD, which were consistent over several repeated separations of *L. anatifera* adhesive. The low number of bands (10-20) seen on each SDS gel suggests that the number of proteins that are involved in *L. anatifera* adhesive is approximately of the order of about 10 proteins, however this remains an imprecise guess. One dimensional SDS-PAGE protein bands can underestimate the number of proteins present when different proteins of the same molecular weight are present, but can also overestimate protein numbers when several bands represent a single protein. Some evidence was seen to support the latter case when identical *de novo* peptide sequences were found in bands of different sizes. This can be counteracted by performing SDS-PAGE in two dimensions, however 2D SDS-PAGE is not compatible with the strong urea/thiourea buffer that was required for solubilisation of *L. anatifera* adhesive.

The appearance of identical *de novo* peptides in multiple bands could have occurred for several reasons: the bands could represent oligomers of a single small protein; each band could contain more than one protein, for instance each may consist of a larger protein with the same smaller protein embedded within; or the different bands may be variants of the same protein. The presence of oligomers is unlikely as the space between each band is not equal. Multiple variants of the same protein are possible but would be expected have much more similar molecular weights. Multiple variants within the same species of the barnacle adhesive protein cp-20k have been observed in *A. amphitrite* (He

et al., 2013) and multiple variants of adhesive proteins have also been observed for tubeworm Pc-3 (Zhao et al., 2005) and mussel fp-3 (Zhao et al., 2006).

A final problem in making comparisons of SDS-PAGE bands is that of abnormal protein migration, which has been reported for the fully characterised adhesive proteins cp-52k and cp-100k (Kamino et al., 2000; Kamino et al., 2012), with discrepancies found between actual molecular weight and SDS-PAGE molecular weight. It is clear that deductions about protein molecular weights from SDS-PAGE bands alone is an inexact exercise, however, some similarities were observed in the protein sizes reported in three recent studies of stalked barnacle species (including the current study): consistent bands between species were apparent at 70, 90, 110 and possibly 150 kD. The variability that existed between the stalked barnacle species was more marked in the smaller protein size ranges.

Attempts to isolate adhesive protein genes using primers based upon *de novo* sequences from the adhesive proteins separated in the current study were unsuccessful. Extensive efforts were made to amplify cDNA sequences using degenerate primers and traditional PCR, as well as RACE PCR, however all amplified products were found to be non-coding cDNA with no open reading frames and no similarity to the *de novo* sequences. The majority of PCR experiments produced no product at all, an unexpected result when using degenerate primers, which generally produce a range of non-specific products. This may indicate that further *de novo* sequencing is required to produce sequence fragments that truly reflect the primary structure of the proteins; when comparing the current results to *de novo* sequences from *D. fascicularis* adhesive (McEvilly, 2011; Zheden et al., 2013) a few similarities were observed, however the similar sequence fragments were not identical. At this point it is impossible to say exactly how accurate each *de novo* sequence fragment may be and much more experimentation will be required to produce accurate amino acid sequences that can be used to isolate adhesive protein genes.

Acorn barnacles have been the source of almost all of our knowledge regarding barnacle adhesive proteins. At least one protein, 20 kD in size, has consistently been reported to be present in acorn barnacle adhesive from various species: 22 kD in *A. eburneus* (Naldrett & Kaplan, 1997), 19 and 20 kD in *M. rosa* and *Fistulobalanus albicostatus*

(Mori et al., 2007; Urushida et al., 2007), 22 kD in *A. improvisus* (Berglin & Gatenholm, 2003) and 20 kD in *A. reticulatus* (Raman et al., 2013). However, protein bands below 30 kD were faint and could not be consistently observed in repeats of 1D SDS-PAGE in the present study. This could be a result of the protocols used in the present study; cp-19k and cp-20k in *M. rosa* make up only a small proportion of the adhesive and could not be separated by the same method as the larger adhesive proteins (Mori et al., 2007; Urushida et al., 2007). This is unfortunate as the evidence to date is that the cp-19k and cp-20k adhesive proteins have been amongst the most tractable proteins described in acorn barnacles; they can be produced using recombinant technology (Mori et al., 2007; Urushida et al., 2007) and therefore examined in a variety of ways. The larger barnacle adhesive proteins that have been characterised, cp-52k and cp-100k, are highly hydrophobic and insoluble, which has hindered attempts to create recombinant forms of these proteins (Kamino et al., 2000).

Also consistent across acorn barnacle species were reports for proteins between 20 and 50 kD. A 36 kD protein was observed in *A. eburneus* (Naldrett & Kaplan, 1997) and a 32 kD protein was found in *A. reticulatus* (Raman et al., 2013); in the present study a protein band at approximately 30 kD was strong and observed consistently over many repeats of SDS-PAGE, in agreement with the aforementioned studies. Similarity between species was also seen in the presence of protein bands between 50 and 70 kD: 52 and 58 kD in *A. eburneus* (Naldrett & Kaplan, 1997), 52 and 68 kD in *M. rosa* (Kamino et al., 2000) and 58 and 64 kD in *A. reticulatus* (Raman et al., 2013). However only faint, inconsistent bands were observed in this range in *L. anatifera* SDS-PAGE experiments, with the strongest band being present at 70 kD. This is in contrast to cp-52k, which makes up a large proportion of acorn barnacle adhesive (Kamino et al., 2000). If a homologous protein exists in *L. anatifera* adhesive, it may have a significantly different mass. A protein of 47 kD was observed in *Dosima fascicularis* (Zheden et al., 2013), however this was not supported by a separate investigation of *D. fascicularis* adhesive (McEvilly, 2011). Finally, a large protein of around 100 kD has been observed consistently in several species (Berglin & Gatenholm, 2003; Kamino et al., 2000; Raman et al., 2013). This is comparable to the 90 and 110 kD protein bands observed in stalked species *L. anatifera* and *Dosima fascicularis* (McEvilly, 2011; Zheden et al., 2013).

While the size and number of proteins indicates that the same substance is being analysed across species, a comparison of adhesive proteins that have been studied in more depth shows that homologous adhesive proteins have low sequence similarity and can differ drastically in molecular weight and pI. In *M. rosa* cp-19k has a pI of 5.8, while in *F. albicostatus* and *A. improvisus* cp-19k has a pI of 10.3 (Urushida et al., 2007). Another small protein, cp-20k, has a molecular mass of 12,297 Da and pI of 8.3 in *F. albicostatus* (Mori et al., 2007) while in *M. rosa* the molecular mass of this protein is 20,373 Da with a pI of 4.72 (Kamino, 2001). These observations indicate that there must be significant sequence differences in these homologous adhesive proteins. A picture that begins to emerge is that, in barnacle adhesive, the functional homology of proteins does not strictly depend on the characteristics of size, pI and primary sequence similarity. The result is that protein identification with MS becomes difficult, and isolation of homologues from more distantly related species with traditional PCR methods is unlikely to be successful. While it is entirely possible that *L. anatifera* possesses adhesive proteins that are homologous to acorn barnacle proteins, these appear to be so distinct that no significant similarity could be found using MS. The other possibility is that the adhesive proteins from *L. anatifera* are not homologous to those of acorn barnacles. As of yet, it is difficult to distinguish between these possibilities; barnacles in general are not well represented in DNA and protein databases, especially in regard to adhesive proteins, adding further hindrance to the identification of the proteins separated during the course of this study. The majority of database entries for various barnacle species were gathered for phylogenetic studies and these do not sufficiently represent functional genes and proteins in these animals. The advent of affordable next generation sequencing, including transcriptome experiments (e.g., Chen et al., 2011), may alleviate some of these gaps.

While the adhesive proteins of *L. anatifera* are clearly distinct from published sequences of acorn barnacle adhesive proteins, similarities can already be seen between *L. anatifera* and the more closely related *D. fascicularis*. The strongest, most consistent protein bands observed in the adhesive of *L. anatifera* were also reported in the adhesive of *D. fascicularis.* From *de novo* sequence fragments it can also be seen that there is primary structure homology between adhesive proteins from the two species (McEvilly, 2011; Zheden et al., 2013). In mussels, fp-1 is conserved across a wide range of species, but the primary sequences of distantly related species cannot be aligned at

all; only when looking at the fp-1 repeated motif from many species together can the relationship between the primary sequences be seen (Anderson & Waite, 1998; Burzio et al., 2000; Clezar et al., 2008; Inoue & Odo, 1994; Inoue et al., 1996; Zhao & Waite, 2005). It is possible that homologous adhesive proteins from barnacles have also developed such distinct primary sequences that their relatedness will only be discovered when a much wider range of barnacle species' adhesives have been examined. The upshot of the great diversity in adhesive protein sequences currently reported for barnacles is that conserved adhesive domains remain difficult to spot and the functional mechanisms of the adhesive remain elusive.

When considering the history of research into barnacle adhesive proteins it is apparent that isolating and characterising these genes is no simple process. The longest standing research in this area comes out of Japan by K. Kamino and co-workers (Kamino, 2001; Kamino et al., 2000; Kamino et al., 2012; Kamino et al., 1996; Urushida et al., 2007). Even in this case, the first paper described the number of proteins present in barnacle adhesive, including N-terminal sequences, in 1996 (Kamino et al., 1996), but it was four years before any full cDNA sequences were published (Kamino, 2001; Kamino et al., 2000), another six until a further adhesive protein was fully characterised (Urushida et al., 2007) and finally the characterisation of a fourth adhesive protein came five years later (Kamino et al., 2012). In 16 years four proteins have been fully characterised out of at least eight possible proteins. In addition, no other research group has continued in this area of work; Naldrett (1993) and Naldrett and Kaplan (1997) proceeded so far as to separate the adhesive proteins by way of SDS-PAGE and publish N-terminal amino acid sequences, however their work stopped there. Recent research in the area of barnacle adhesive has focused on different aspects and not attempted to characterise the protein composition of the adhesive or sequence further adhesive proteins or their homologues from various species, yet it is clear that to fully understand barnacle adhesive, proteins from a wide range of species will need to be characterised. The novel adhesive proteins present in the stalked barnacle *L. anatifera* exemplify how much more there is to discover about barnacle adhesive.

5.5 References

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6 Barnacle adhesive proteins: conservation and localisation

6.1 Introduction

6.1.1 Conservation of barnacle adhesive proteins

Adhesion is achieved in all barnacles by a complex of proteins that work together to form strong bonds with the substrate, each other and the body of the barnacle. Sequence information is available for four barnacle adhesive proteins but despite this information being available for several years now, the mechanisms involved in the adhesion, cohesion and setting of barnacle adhesive are unknown. The initial characterisation of the barnacle adhesive proteins, cp-19k, cp-20k, cp-52k and cp-100k, was carried out in the acorn barnacle *Megabalanus rosa* (Kamino, 2001; Kamino et al., 2000; Kamino et al., 2012; Urushida et al., 2007). Homologues have now been isolated in further acorn barnacle species; amino acid compositions are similar between species, however sequence identity (percentage of identical amino acids) between the homologues is not high (Mori et al., 2007; Urushida et al., 2007). While sequence is not highly conserved, homologues of cp-19k share an amino acid composition that consists of a high proportion of Ser, Thr, Ala, Gly, Val and Lys (Urushida et al., 2007), and has been suggested to be arranged in alternating blocks: Ser-Thr-Gly-Ala rich and Val-Lys rich (Kamino, 2008). In cp-20k there is a bias for Cys residues, which are arranged in a regular, repeating pattern that creates a novel three-dimensional structure supported by disulfide bonds and β-hairpins (Suzuki et al., 2005). Similar repeating units have been reported in homologues from four acorn barnacle species, indicating that this structural motif is important for the function of cp-20k (He et al., 2013; Mori et al., 2007). As more barnacle adhesive protein sequences become available, closer examination of any conserved characteristics may be vital for the identification of the elusive adhesive mechanisms utilised by barnacles.

6.1.2 Conservation of adhesive proteins from other marine animal models

The mussel adhesive protein foot-protein 1 (fp-1) largely consists of a repeated sequence motif; fp-1 of *Mytulis edulis* contains about 70 repeats of a decapeptide containing L-3,4-dihydroxyphenylalanine (DOPA) and hydroxyproline (Filpula et al., 1990). Homologues of fp-1 have been isolated in a wide range of species, providing insight into the relationship between sequence structure and protein function. While

some closely related species have the same or a very similar decapeptide repeated in fp-1, there are vast differences between genera (Table 6.1). The relationship between the fp-1 homologues can be seen when the repeated motifs are directly compared, whereas attempting to align the cDNA sequences of the homologues conceals the similarity between the proteins. It is clear that the function of fp-1 relies on the repetition of short motifs rich in lysine, proline, serine, tyrosine and threonine and the presence of the redox-active DOPA. Yet even DOPA is not the defining characteristic of fp-1; in *P. viridis* tyrosine has been replaced by glycosolated tryptophan and DOPA has been mimicked by hydroxyl-tryptophan (Zhao et al., 2009).

Table 6.1: Repeating amino acid motifs appearing in fp-1 of various mussel species. *-Ppfp-1 is an incomplete sequence and is only assumed to be a repeating motif.

Species	Amino acid repeat sequence	Reference
Mytilus edulis	A K P S Y P P T Y K	(Filpula et al., 1990)
M. galloprovincialis	A K P S Y P P T Y K	(Inoue & Odo, 1994)
Perna perna *	K P S Y P P T	(Clezar et al., 2008)
M. californianus	P K I S Y P P T Y K	(Holten-Andersen et al., 2009)
M. coruscus	P K I/P S/T Y P P T/S Y K	(Inoue et al., 1996)
Choromytilus chorus	A K P S Y P T G Y K P P V K	(Burzio et al., 2000)
P. viridis	A T P K P W T A W K	(Zhao et al., 2009)
	A P P P A W T A W K	
P. canaliculus	P Y V K	(Zhao & Waite, 2005)
Aulacomya ater	A G Y G G V K	(Burzio et al., 2000)
Trichomya hirsute	S Y Y P K	(Zhao & Waite, 2005)
Modiolus modiolus	S S Y Y P K	(Zhao & Waite, 2005)

There is relatively little information available regarding the adhesive substance of other marine animals, leaving the homology and conservation of adhesive proteins to be largely guessed at. Amongst polychaetes at least 14 adhesive proteins (cement proteins) have been isolated from *Phragmatopoma californica* (Endrizzi & Stewart, 2009) but only four of these have been looked for in a different species. The cement proteins 1, 2, 3A and 3B have now been isolated in *Sabellaria alveolata* (Becker et al., 2012) and were found to be similar to the extent that primers based on *P. californica* cDNA sequences were successful, but different enough that alignment of the complete sequences was very poor. The proteins had similar characteristics, however the underlying sequences showed significant divergence. In contrast, the conservation of echinoderm adhesive components has thus far only been studied through

immunohistochemical analyses; no adhesive protein primary structures or cDNA sequences have been published. It has been reported that while sea star tube foot adhesive from a range of orders, families and species is closely related and can be visualised with footprint antibodies raised from *Asteria rubens* (Santos et al., 2005), the tube foot adhesive of sea urchins is likely to be species specific, as antibodies raised from the footprints of one species had no reaction to the tube feet of other species (from different orders and families) (Santos & Flammang, 2012).

6.1.3 Barnacle adhesive protein production

The second topic of interest in this chapter is localising adhesive protein expression. The relevance of this topic has resurfaced recently after suggestions of a two-step adhesion process (Burden et al., 2012). The adhesive production system of all barnacle taxa is characterised by a proteinaceous adhesive, produced in large cells that are isolated from one another and drained by paired canals to the substratum (Chapter 3; Lacombe, 1970; Lacombe & Liguori, 1969). This system is vastly different to the multi-cellular, multi-glandular systems used by animals such as tubeworms and mussels (Brown, 1952; Silverman & Roberto, 2010; Wang et al., 2010). Recent structural investigations support the assumption that the adhesive gland cells are responsible for a single, homogenous adhesive secretion (Jonker et al., 2012; McEvilly, 2011; Zheden et al., 2012), but it has recently been suggested that there are two different kinds of adhesive secretion (Burden et al., 2012). This most recent study introduces the possibility of a paradigm shift for researchers in this field, suggesting a multi-step process with possible enzyme involvement or hitherto unknown cross-linking mechanisms, bringing the barnacle adhesive production process more in line with what is known from mussel and tubeworm adhesives. Optical and structural experiments by Burden et al. (2012) indicate that the bulk of the adhesive (cement) secretion (BCS1) is secreted regularly at the periphery of the barnacle base plate/adhesive plaque. A second adhesive secretion (BCS2) is secreted discontinuously and adds a significant level of strength to the adhesive, either through its own adhesive processes or by interacting with the bulk of the adhesive to create a fibrillar network and intermolecular cross-links (Burden et al., 2012). The authors suggest that phenolic compounds may yet play a role in the barnacle adhesive, despite past evidence against this hypothesis (Barnes & Blackstock, 1976; Naldrett, 1993). Currently, internal structural information does not

support the suggestions of Burden et al. (2012), thus examination of patterns of adhesive protein expression are an important next step to provide evidence for or against the presence of a second adhesive secretion.

Previous studies have used northern and western blotting to confirm that the adhesive protein genes thus far characterised are expressed in the basal portion of acorn barnacles, which is where the glands are located, while the upper part of the body and shell did not express the adhesive proteins (Kamino et al., 2000; Kamino et al., 2012). However, the basal part of the acorn barnacle body also contains the ovaries, connective tissue, muscles and cuticle, all located within a very small area, so localising adhesive protein expression using this method is not conclusive. The stalked barnacle *Lepas anatifera* is a good candidate to investigate localised expression patterns because it is large-bodied relative to most of the acorn barnacle species and the adhesive glands are spread out and relatively easy to distinguish from other tissues. It is furthermore possible to use *L. anatifera* as an example of a distantly related taxon in which to examine conservation of adhesive proteins within the barnacles as a group.

6.1.4 Aims

The primary aim of this study was to investigate the conservation of barnacle adhesive proteins in *Lepas anatifera* (order Lepadiformes) using antibodies raised against adhesive proteins from acorn barnacles (order Sessilia). Specifically, the presence of cp-52k, cp-68k and cp-100k was investigated in *L. anatifera* tissue and their localised expression examined. In addition, PCR methods were used to examine whether barnacle adhesive protein homologues (cp-19k, cp-20k and cp-100k) could be isolated from *L. anatifera*.

6.2 Materials and methods

6.2.1 Immunohistochemistry

Samples of *Lepas anatifera* and *Amphibalanus improvisus* were fixed in either Bouin's fluid (both a fixative and decalcifier) or 4% PFA in PBS, dehydrated and embedded in paraffin. Sections of 5 µm were cut and placed on SuperFrost Plus (Thermo Scientific) slides. Sections were deparaffinised, rehydrated and moved into TBS (tris-buffered saline) (pH 7.6). Antigen retrieval was performed by placing sections in a sodium citrate buffer (10 mM sodium citrate, 0.05% Tween, pH 6.0) and heating to 98 °C for 20 minutes. After sections had cooled they were incubated in 3% H_2O_2 for 30 minutes, then non-specific binding was blocked by incubation in 4% normal goat serum for 2 hours at room temperature. Polyclonal antibodies against cp-52k, cp-68k and cp-100k from *M. rosa* were provided by Professor K. Kamino. These antibodies (raised in rabbits) were diluted to 1 in 1000 in blocking solution. Primary antibodies were applied to sections and incubated overnight at 4 °C. After washing in TBS with 0.025% Triton, the Rabbit ExtraAvidin Peroxidase staining kit (Sigma Aldrich) was applied according to the manufacturer's directions. Colour was developed with AEC chromogen (Sigma Aldrich) for 5 minutes, then washed in running tap water for 5 minutes. Coverslips were mounted using Aquatex (Merck Millipore) and slides were examined with an Olympus BX51 compound microscope. The acorn barnacle *A. improvisus* was used as a positive control, as it has been shown that the adhesive proteins cp-100k and cp-68k are conserved in acorn barnacles and present in *Amphibalanus* species (Kamino, 2008). At this point in time only a single published sequence of cp-52k is available, from *Megabalanus rosa*, however it is assumed that the adhesive protein is also present in other species of barnacle. Negative controls were processed alongside each experiment, without the addition of the primary antibody.

6.2.2 Degenerate primer design

Isolation of adhesive proteins in *L. anatifera* was attempted using degenerate primers based on unpublished sequences, along with the published barnacle adhesive sequences available from the NCBI database (Table 6.2). The only published sequence for cp-100k, from *Megabalanus rosa*, was used in conjunction with sequences for *A. amphitrite* and *Fistulobalanus albicostatus* provided by Professor K. Kamino. An NCBI

tBLASTx search for homologues to cp-100k produced EST sequences from *Pollicipes pollicipes* (Meusemann et al., 2010) that could be aligned with parts of the cp-100k gene and used to locate potential primer sites. Other *P. pollicipes* EST sequences were aligned with parts of the cp-19k gene but *P. pollicipes* EST similarity with cp-19k was not high enough to present suitable primer sites.

Table 6.2: Barnacle adhesive protein sequences available in the NCBI database.

Sequence	Accession No.
M. rosa cp-100k	AB633742
F. albicostatus cp-100k	Unpublished
A. amphitrite cp-100k	unpublished
P. pollicipes cp-100k EST	FN246186
	FN544618
	FN546751
	FN246068
	FN244667
	FN244663
M. rosa cp-20k	AB035415
F. albicostatus cp-20k	AB329666
M. rosa cp-19k	AB242294
F. albicostatus cp-19k	AB242295
A. improvisus cp-19k	AB242296
P. pollicipes cp-19k EST	FN244142

Both nucleotide and amino acid sequences were aligned using ClustalW through the software programmes MEGA5 (Tamura et al., 2011) and GeneDoc (Nicholas et al., 1997). Primers were then designed manually based on the most conserved sections of sequence. To reduce the degeneracy of the primers as much as possible primers were designed based on the nucleotide sequences (Table 6.3). Further primer sequences were also supplied by Professor K. Kamino. All PCR reactions were run with a positive control, using primers for 18s ribosomal DNA, and negative controls containing no *L. anatifera* DNA.

Table 6.3: Primers created from alignments of cp-19k, cp-20k, cp-100k and 18s (control), reverse primers are written as reverse complement sequence. F-forward, R-reverse, P-primers from K. Kamino, pol-primers created from alignment including *P. pollicipes* EST sequences.

Primer	Sequence
18s F	atggccgttcttagttggtg
18s R	gcgctgaccacttcagtgta
Cp-19k F1	gtgccvccrccrtgcgac
Cp-19k F2	ccrcrtgcgacytyrgcatc
Cp-19k F3	rgcrccacsmrsggctccggc
Cp-19k R1	kccgwysgkgagmaggtc
Cp-19k R2	sccrccrttrgcaycvgc
Cp-19k R3	vswccggagscctkgtg
Cp-20k F1	rtcmtkgcrrcakttgtc
Cp-20k F2	arysmcccstgctaccactgc
Cp-20k R1	rtgwtyctsrttgcacgg
Cp-20k R2	gcakrwmcagncrcrgygctt
Cp-100k F1	cagtcsagctacagcatg
Cp-100K F2	gtcscagggyatycagcc
Cp-100k F3	tmcaarctyctyccgaag
Cp-100k R1	gtrctgswcgasgggcgg
Cp-100k R2	ctcctgratnayytckac
Cp-100k R3	racgttytsgtactcraa
Cp-100k P3	aayathytngtncc
Cp-100k P3r	ggnacnardatrtt
Cp-100k P7	ggntaygtnathccnca
Cp-100k P7r	tgnggdatnacrtancc
Cp-100k P8r	atnardatngtrtg
Cp-100k-pol F1	cgchdhkgctgcggctg
Cp-100k-pol R1	gacbysscgctgrtgcag
Cp-100k-pol F2	ggvcyrcarggmmrcagc
Cp-100k-pol R2	gwsrymacgwagaactg

6.2.3 RNA extraction, cDNA synthesis and PCR

RNA was extracted from tissues stored in RNAlater (Qiagen) at -70 °C using the RNeasy kit with Qiashredder columns (Qiagen) according to manufacturer's instructions for purification of total RNA from animal tissues. RNA quality was tested on a 1.5% agarose formaldehyde gel (clear bands without smearing indicate clean RNA) and a NanoDrop spectrophotometer was used to both check for purity and

concentration. RiboLock RNase inhibitor (Thermo Scientific) was added to the RNA solution prior to storage at -70 °C.

Extracted RNA was used to synthesise cDNA with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Concentration of cDNA was determined using a NanoDrop and the cDNA was diluted to 0.25 µg/µl with pure H_2O before being stored at -20 °C.

PCR reactions were conducted with GoTaq (Promega) according to Table 6.4 and Table 6.5. PCR reactions were run on a 1% agarose gel containing SybrSafe (Invitrogen) at 150 V for 25 minutes. Gel bands of the desired size were carefully removed with a clean scalpel and placed in clean 1.5 ml eppendorf tubes, after which they were either stored at -20 °C or immediately purified.

Table 6.4: Components of the PCR reaction as used for all PCR reactions in this study.

Reagent	Concentration	Volume (µl)
GoTaq flexi buffer	5X	10
DNTPs	10 mM	1
MgCl	25 mM	5
GoTaq	5 units/µl	0.25
cDNA	0.25 µg/µl	2.5
Sense primer	10 µM	2
Antisense primer	10 µM	2
H_2O		27.25

Table 6.5: Protocol for PCR reaction.

	Step	Temperature (°C)	Time
	Initial denature	94	2 minutes
35 cycles	Denature	94	30 seconds
	Annealing	48-62	30 seconds
	Extension	72	1 minute
	Final extension	72	5 minutes

6.2.4 Ligation, transformation and cloning

The PCR amplified products were purified from gel bands using the QIAquick gel clean-up kit (Qiagen) according to the manufacturer's protocol, using water to elute the DNA from the spin column in the final step. The purified product was either stored at -20 °C or immediately ligated and transformed. The vector pCR-II-TOPO (Invitrogen) and TOP10 competent *E. coli* cells were used for all experiments, according to the manufacturer's protocol. Transformed cells were divided and spread onto two pre-warmed agar plates and incubated overnight at 37 °C.

Blue and white screening was used to identify colonies containing a vector insert. For each amplified product 10 white clones were picked from the plates and grown in a shaking incubator for 8 hours in 1 ml of LB broth at 37 °C, 200 rpm. Clones were screened via PCR using M13 sense and antisense primers, according to the protocols outlined in Table 6.4 and Table 6.5. Clones that contained inserts of the correct size were grown for a further 8 hours in 6 ml LB broth at 37 °C, 200 rpm. Plasmids were extracted using the PureYield plasmid miniprep system (Promega) according to the manufacturer's protocol. The concentration of the purified plasmid was measured on a NanoDrop and the plasmid inserts were subsequently sequenced using either the T7 or SP6 primers by GATC-biotech.

6.2.5 Sequence analysis

The adhesive protein sequences were analysed through a combination of free access online software. Alignments were created with the programmes MEGA5 (Tamura et al., 2011) and GeneDoc (Nicholas et al., 1997). Protein characteristics were examined with Protparam (Expasy) and Pepstats (Emboss). The sequenced products of PCR experiments were subjected to ORF-finder (NCBI), Translate-tool (ExPasy) and various Blast (NCBI) searches.

6.3 Results

6.3.1 Immunohistochemistry

Polyclonal antibodies against adhesive proteins from *Megabalanus rosa* were examined to investigate whether they produced any localised cross-reactivity in the adhesive gland of *Lepas anatifera*. Antibodies against cp-52k, cp-68k and cp-100k were previously used successfully for western blotting (Kamino et al., 2012 and K. Kamino 2013, pers. comm., 20 Jan.). The acorn barnacle *A. improvisus* acted as a positive control.

The cp-52k antibody (ab-cp-52k) had a positive reaction to the adhesive gland cells of both *A. improvisus* and *L. anatifera*, with the gland cells of the various samples staining to varying degrees of red (Figure 6.1). However, some samples stained only very faintly, hardly distinguishable from the negative control. Not only that, but the antibody was not entirely specific, with some small cells within the ovarian tubules also showing a positive reaction in both *L. anatifera* and *A. improvisus*. Sections of the canal system were present in one sample of *A. improvisus* but this showed very little to no reaction to ab-cp-52k. The most important observation to be made from these results is that ab-cp-52k is not entirely suitable for immunohistochemistry because it was non-specific to adhesive gland tissue in both species. However, despite some positive reaction in the ovarian cells it does appear that ab-cp-52k shows the presence of cp-52k in the adhesive glands of both *A. improvisus* and *L. anatifera*. Cp-52k appears to be homogenously spread throughout the cytoplasm of the adhesive gland and is not present in the nucleus, nor in what appear to be large, empty ‘vacuoles’ (see chapter 3 for detailed description of adhesive glands).

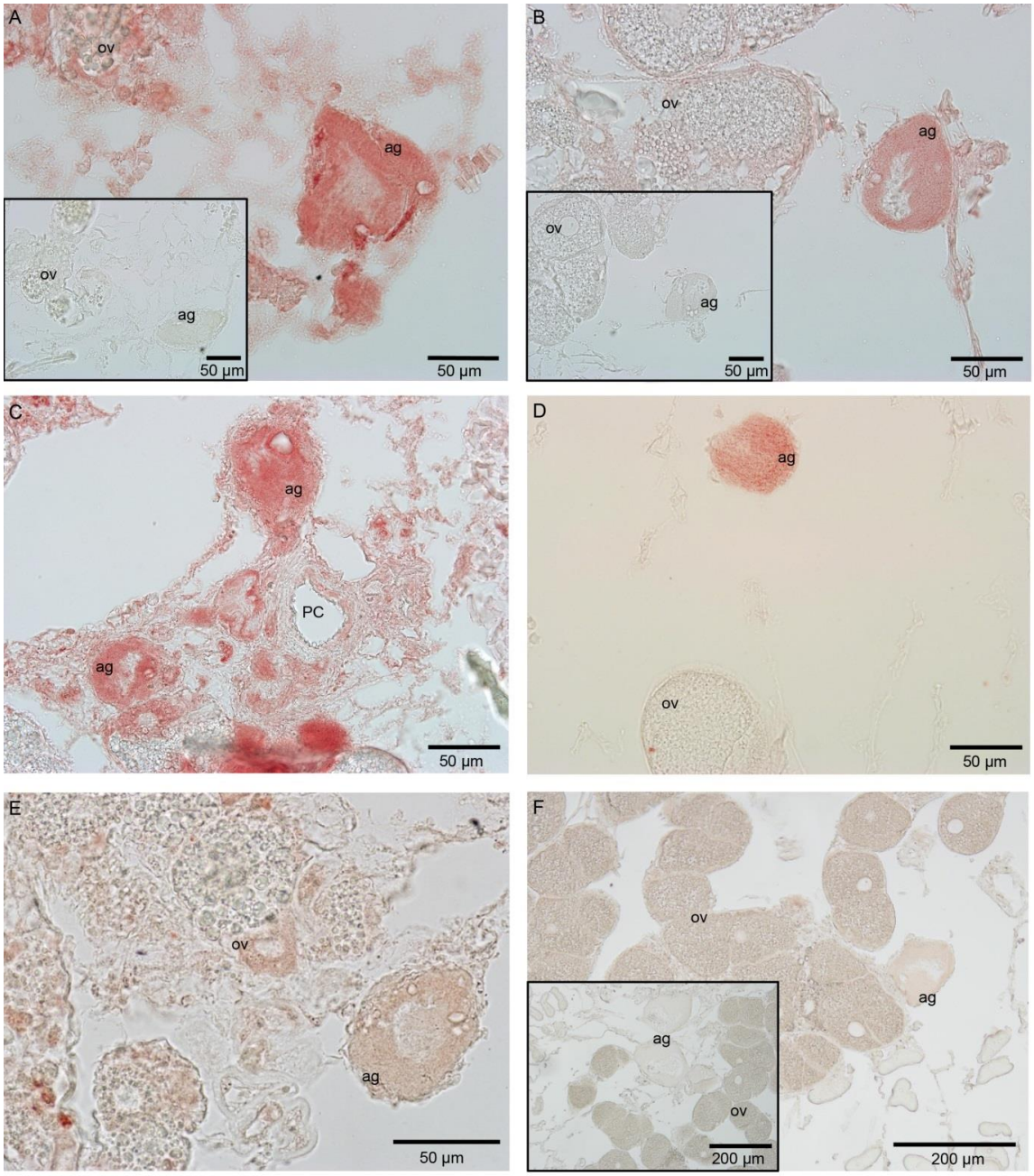


Figure 6.1: Immunohistochemistry for cp-52k with a polyclonal antibody. A, C & E) *A. improvisus*. B, D & F) *Lepas anatifera*. Insets are negative control. Ag-adhesive gland, ov-ovarian tubules, PC-principal canal.

The results of immunohistochemical assays with ab-cp-68k showed a positive reaction in the adhesive gland cells, with the adhesive glands of *A. improvisus* being stained intensely red and the adhesive glands of *L. anatifera* showing a more moderate reaction (Figure 6.2). Unlike cp-52k, the reaction in the adhesive gland cells treated with ab-cp-68k was not always homogenous, instead patches of intense colour were observed around the nucleus in some samples. However, ab-cp-68k was also not specific to the adhesive glands alone, with some ovarian cells staining quite intensely red in *A. improvisus* sections, a reaction that appeared to be localised in the nuclei of the ovarian

cells. The overall reaction of *L. anatifera* sections to ab-cp-68k was more muted than that of *A. improvisus*. Again the ovary showed small patches of positive reaction. In a single sample of *L. anatifera* there was no reaction to ab-cp-68k, for unknown reasons. Similar conclusions can be drawn from these results as from the ab-cp-52k results; the antibody is not entirely suitable for immunohistochemistry, yet the results do indicate that cp-68k is present in the adhesive gland cells of *A. improvisus* and *L. anatifera*, and possibly present in very small amounts in some cells surrounding the principal canal.

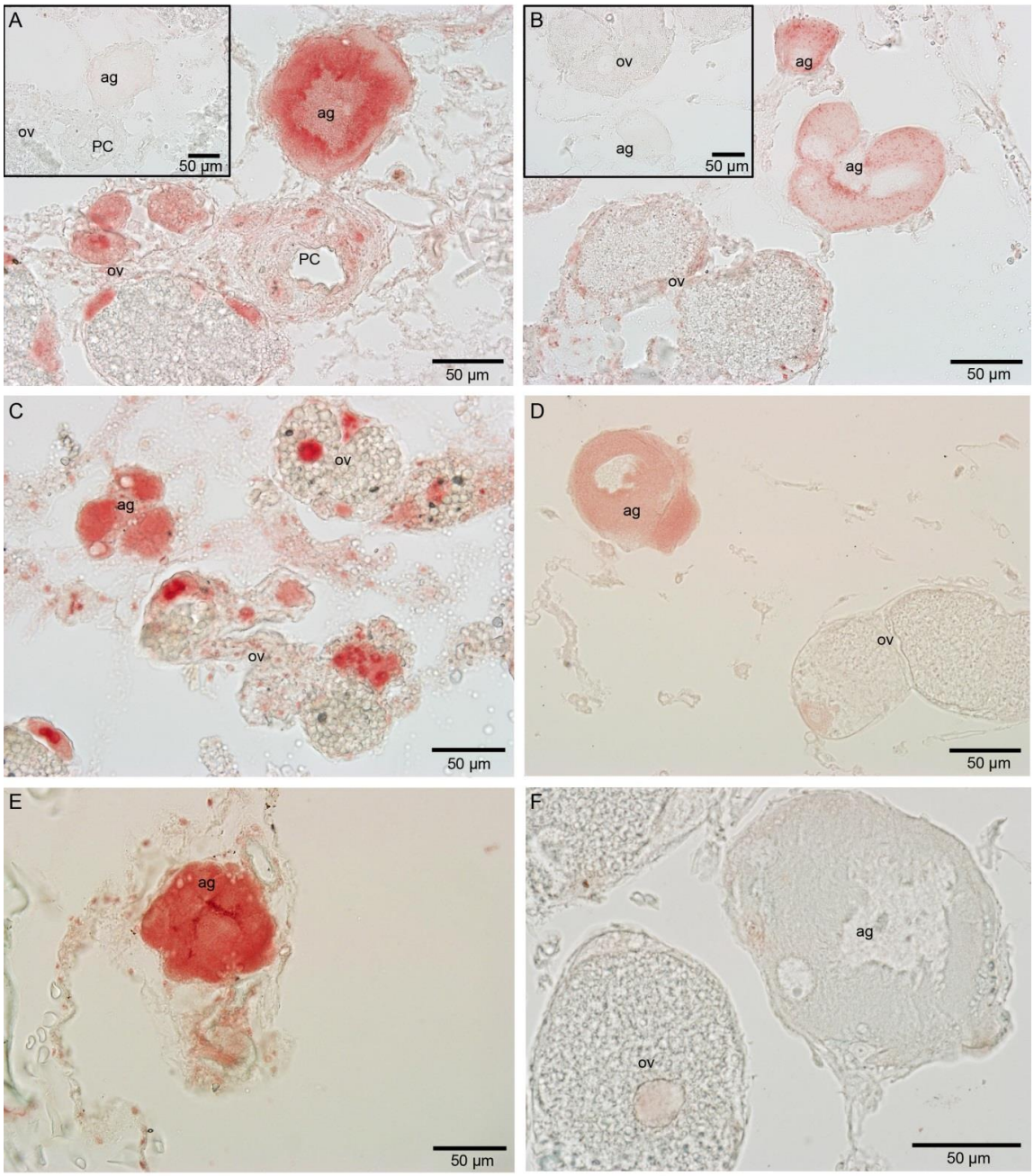


Figure 6.2: Immunohistochemistry for cp-68k with a, polyclonal antibody. A, C & E) *A. improvisus*. B, D & F) *Lepas anatifera*. Insets are negative controls. Ag-adhesive gland ov-ovarian tubules PC-principal canal.

Immunohistochemistry with ab-cp-100k gave the most unexpected result: the adhesive gland cells only reacted very weakly with the antibody, while a strong reaction was seen in patches of the ovarian tubules of both *A. improvisus* and *L. anatifera*. Though some of the staining of the ovary appears to be more brown than red it is clear that ab-cp-100k is entirely unsuitable for immunohistochemistry. No assumptions about the presence of cp-100k in the adhesive gland cells can be made based on this antibody.

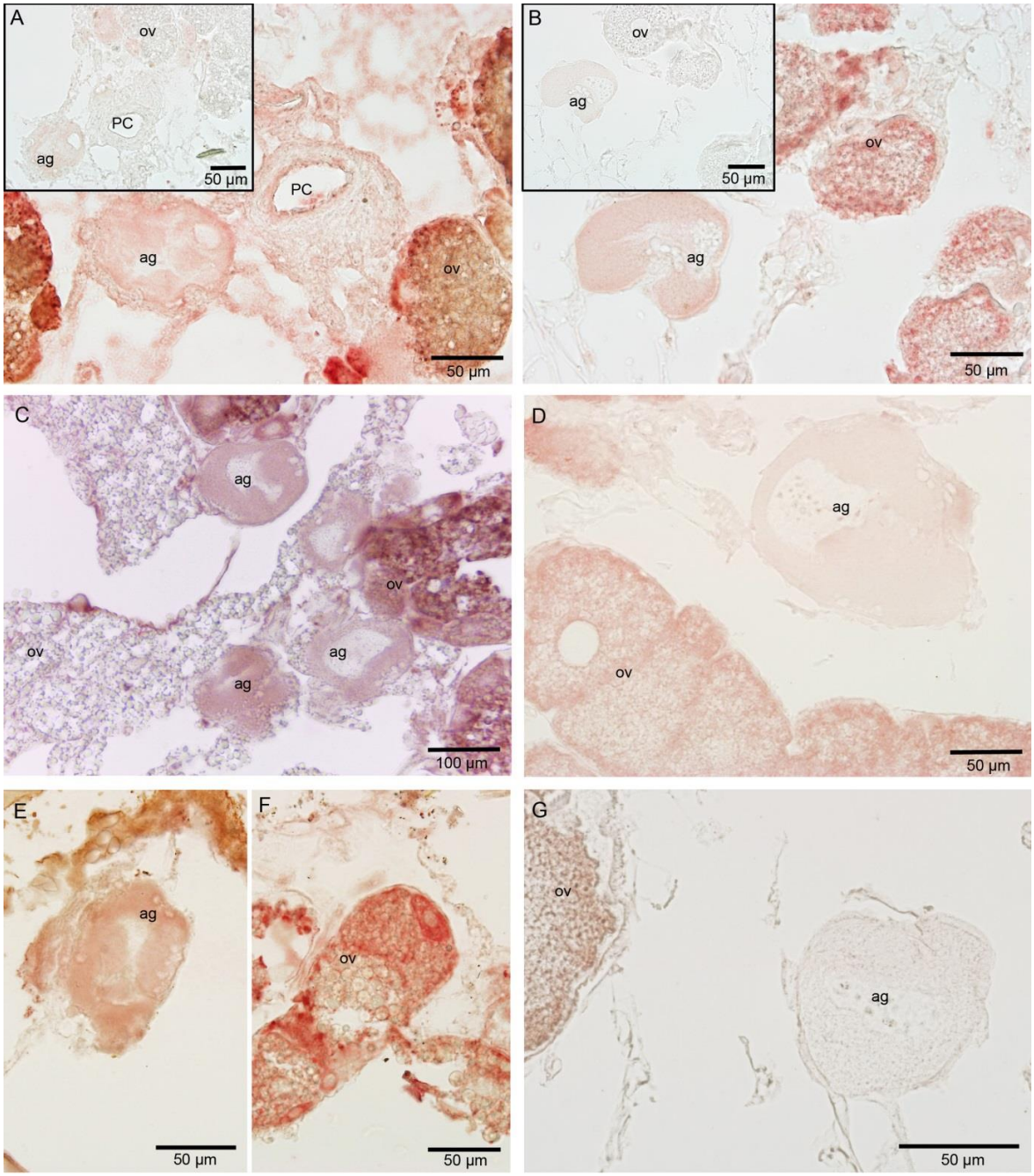


Figure 6.3: Immunohistochemistry for cp-100k using a polyclonal antibody. A, C, E & F) *A. improvisus*. B, D & G) *Lepas anatifera*. Insets show negative control. Ag-adhesive glands, ov-ovarian tubules, PC-principal canal.

6.3.2 Sequence alignment and primer design

Cp-19k sequences were available for three species; initial alignment was performed with amino acid sequences and areas of conservation were identified (Figure 6.4). Sequence identity between *A. improvisus* and *F. albicostatus* was 60%, while sequence identity between *A. improvisus*, *F. albicostatus* and *M. rosa* was 51-54%. The molecular weights of the three proteins were similar, however the pI of *M. rosa* was 5.2, vastly different from the pI of *A. improvisus* and *F. albicostatus* at 9.8. Despite the huge difference in pI the proteins of the three species had very similar amino acid compositions, with a high proportion of Ala, Gly, Lys, Thr, Ser and Val.

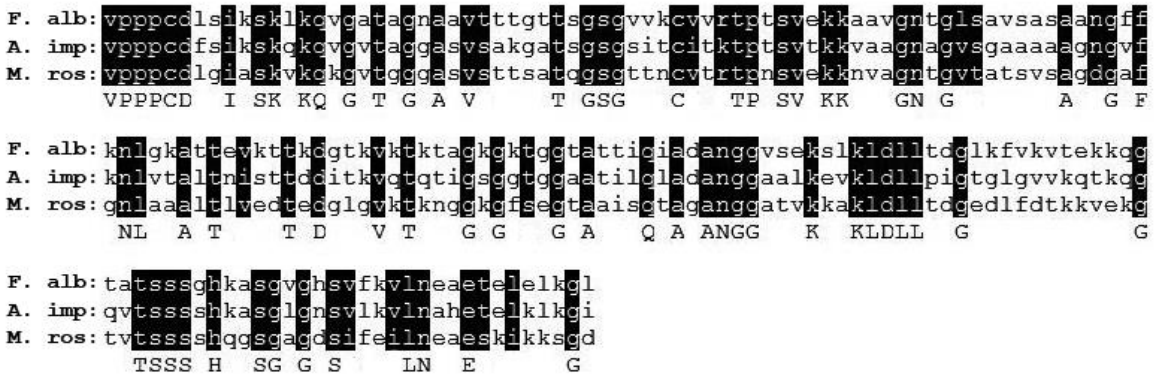


Figure 6.4: Pairwise alignment of entire cp-19k amino acid sequences from *F. albicostatus*, *A. improvisus* and *M. rosa*. Amino acids that are identical or have very similar chemical characteristics are highlighted in black. Consensus sequence is included as the last line of each row.

In order to reduce the redundancy of the degenerate primers a second alignment was performed with cDNA sequences and the addition of a *P. pollicipes* EST sequence. Overall identity between the cDNA sequences was 49% between *A. improvisus* and *F. albicostatus* and 36-40% identity between *A. improvisus*, *F. albicostatus* and *M. rosa*. The *P. pollicipes* EST was 26-36% identical to acorn barnacle species (Figure 6.5). The low sequence identity limited the number of primers that could be designed.

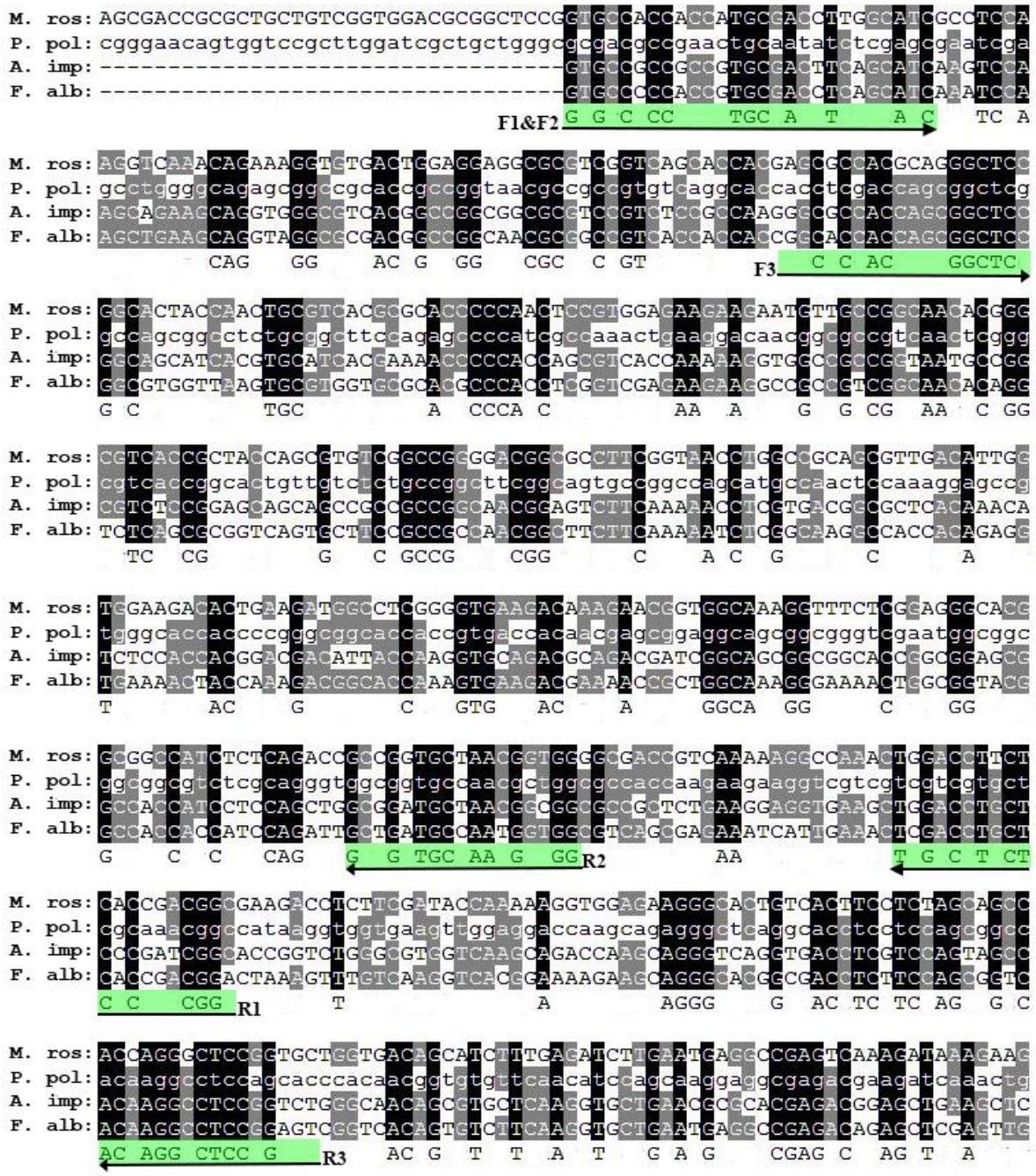


Figure 6.5: Extract from clustal alignment of entire cp-19k genes from *M. rosa*, *A. improvisus* and *F. albicostatus* and EST sequences from *P. pollicipes*. Sequence conservation across four species is shown in black and across three species only is shown in grey. Primer locations shown in green; arrows indicate primer direction. Dashed lines show base pairs that are not present in a sequence. Consensus sequence is included as the last line of each row.

The barnacle adhesive protein cp-20k had the least amount of sequence information available and sequence conservation was low. This adhesive protein has been characterised as having a repeating sequence motif featuring Cys residues, however creating an alignment of the two available amino acid sequences with the cysteine residues aligned (as shown by Kamino (2001)) showed very low sequence conservation, with only 21% identity (Figure 6.6). Both proteins contained a high proportion of Cys

(*F. albicostatus* 14.4% and *M. rosa* 15.8%) however in other aspects the amino acid composition was quite disparate and, as seen in cp-19k, the pI differed between the two genera (*Fistulobalanus* 8.9, *Megabalanus* 4.9). *F. albicostatus* cp-20k had a high proportion of His (16.8%) and other basic residues, with very few acidic residues, while *M. rosa* had less basic residues, 9.4% His and overall was slightly more acidic.

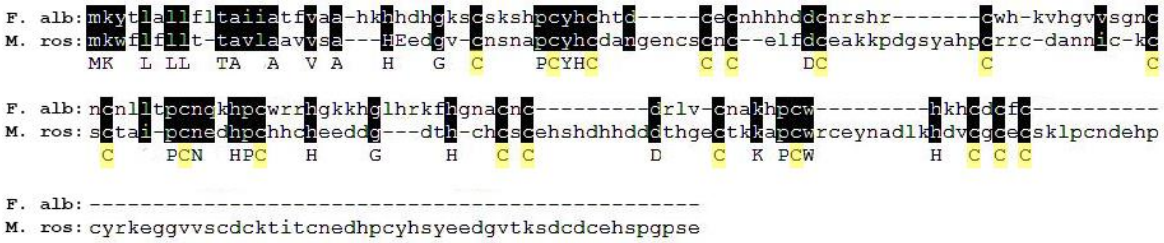


Figure 6.6: Alignment of entire cp-20k amino acid sequences from *F. albicostatus* and *M. rosa*, with Cys residues aligned (yellow highlight) according to Kamino (2001). Amino acids that are identical or have very similar chemical characteristics are highlighted in black. Dashed lines show residues that are not present in a sequence. Consensus sequence is included as the last line of each row.

Using the cDNA sequences for cp-20k a slightly higher identity was seen when the two sequences were aligned (34%), however possible sites for primer design were limited (Figure 6.7).

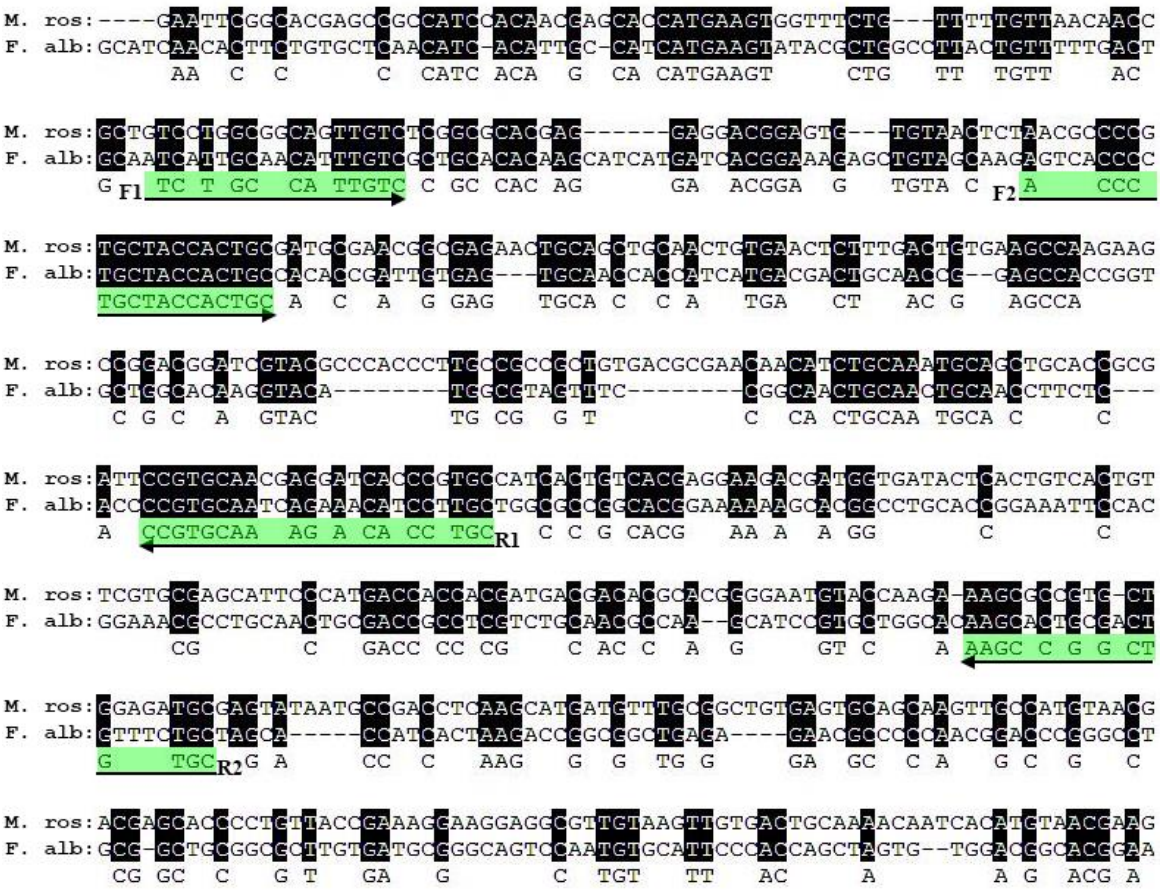


Figure 6.7: Extract from clustal alignment of entire cp-20k genes from *M. rosa* and *F. albicostatus* with sequence conservation shown in black and primer locations shown in green; arrows indicate primer direction. Dashed lines show base pairs that are not present in a sequence. Consensus sequence is included as the last line of each row.

Amino acid sequences of cp-100k for *A. amphitrite*, *F. albicostatus* and *M. rosa* were easily aligned and showed many potential primer sites (Figure 6.8). A closer look at the alignment showed that the two *A. amphitrite* and *F. albicostatus* species shared 64% identity, but *M. rosa* had only 42% identity to the others. Conserved areas featured many highly degenerate amino acids, such as Ser, Leu and Val, thus making poor primer sites. Overall, Ile, Leu, Ser and Val were the most highly represented amino acids, each representing approximately 10% of the residues in the protein.

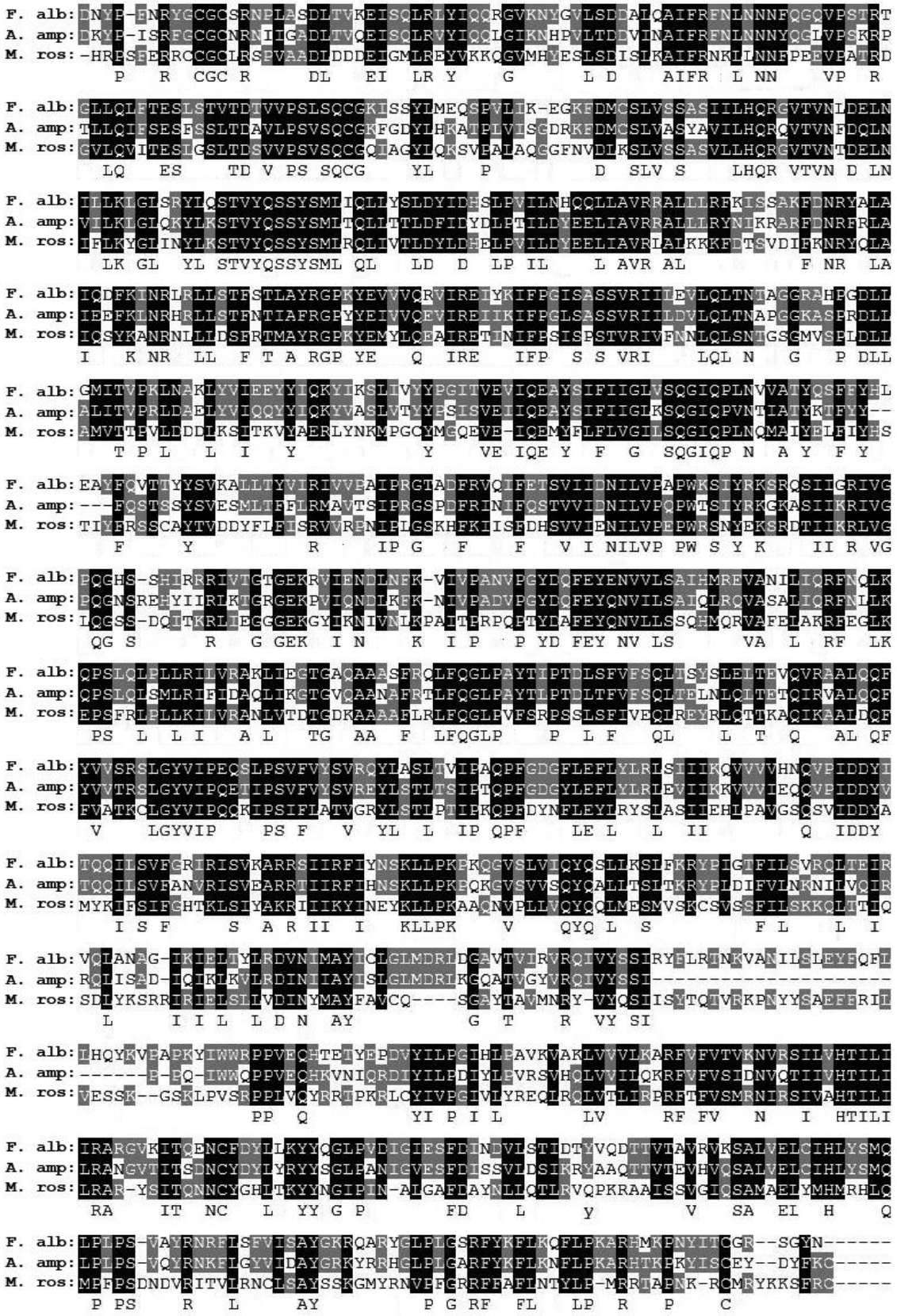
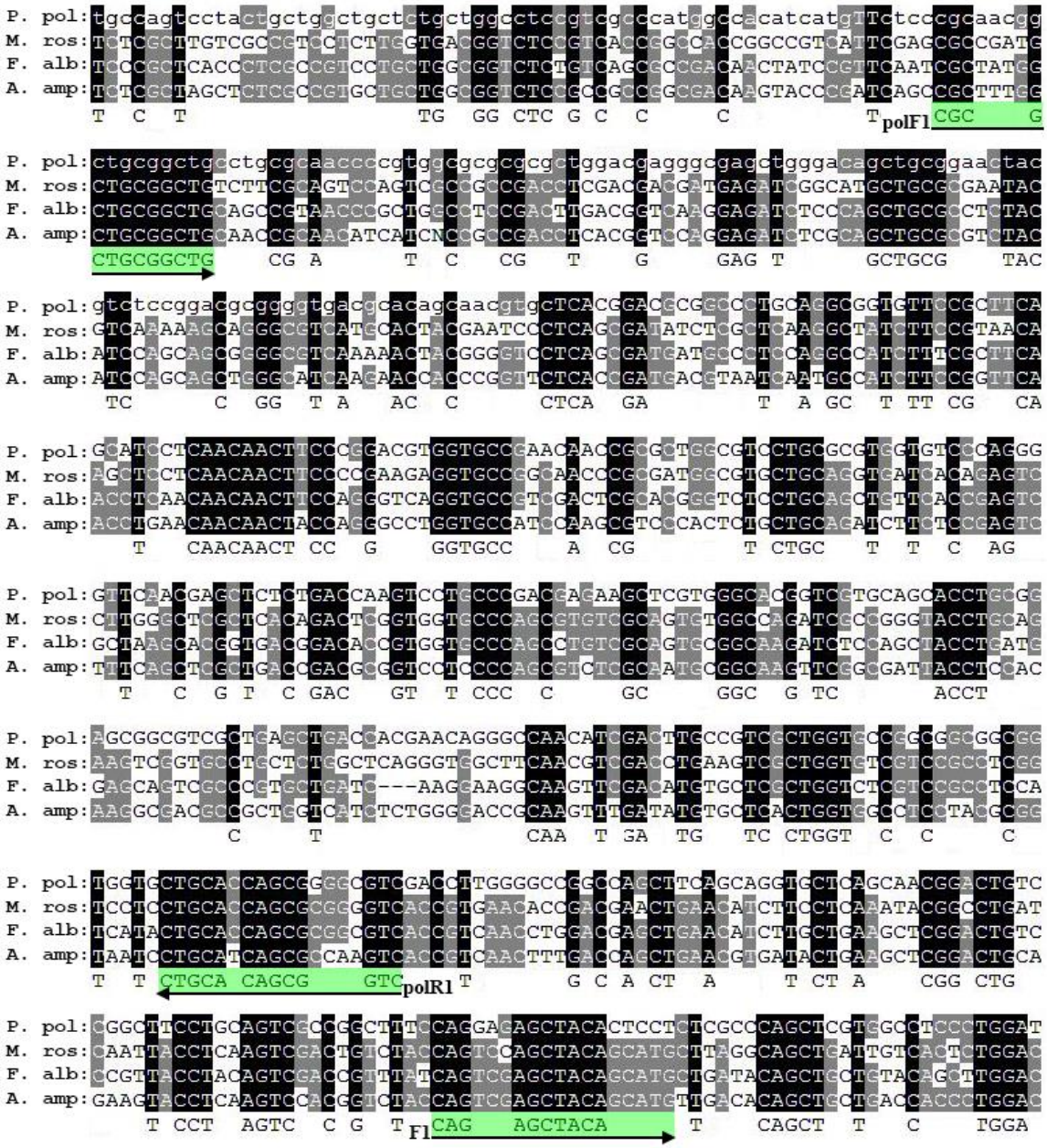
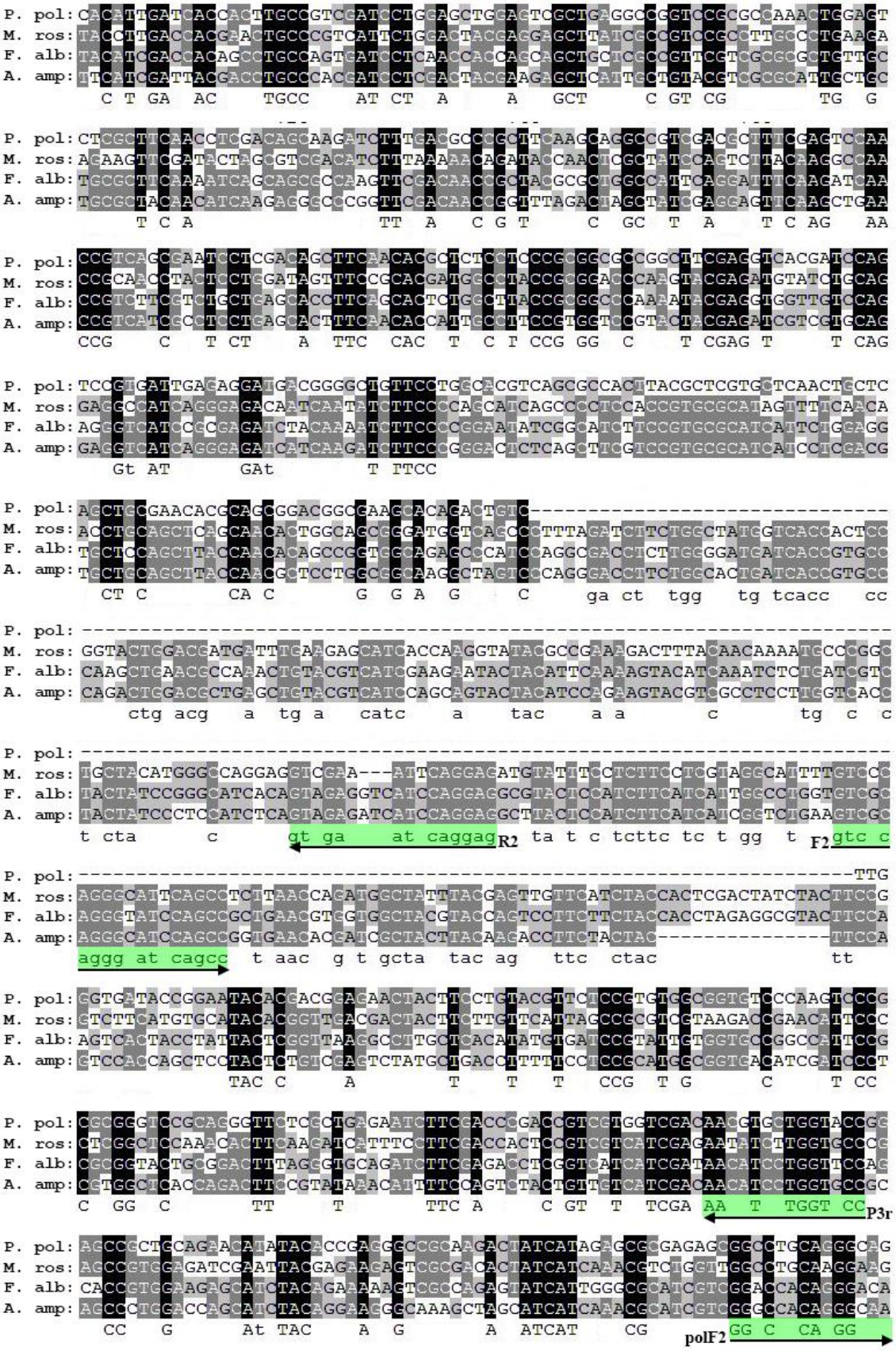
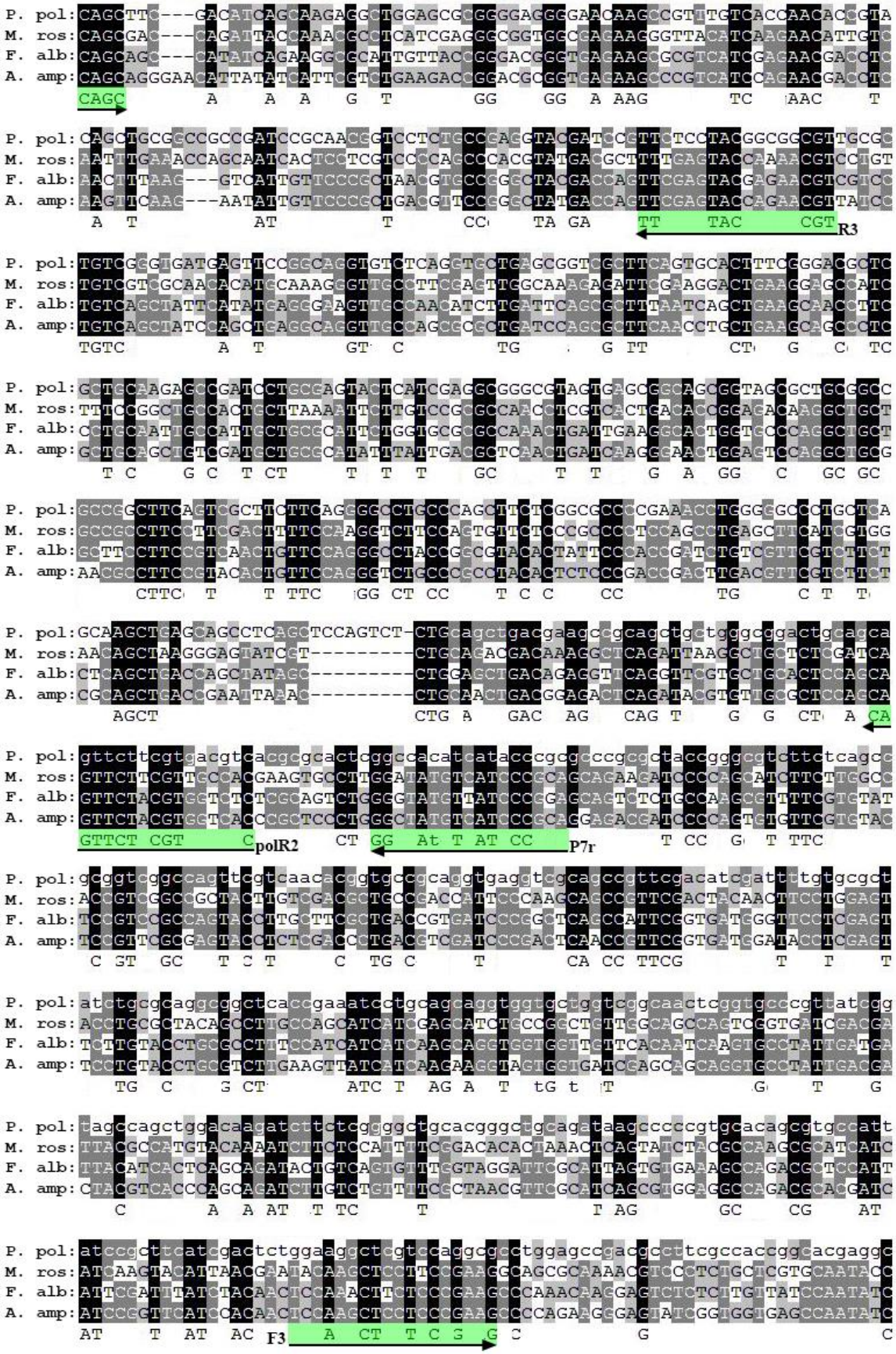


Figure 6.8: Pairwise alignment of entire cp-100k amino acid sequences from *F. albicostatus*, *A. amphitrite* and *M. rosa*. Amino acids that are identical or have very similar chemical characteristics are highlighted in black. Dashed lines show residues that are not present in a sequence. Consensus sequence is included as the last line of each row.

In order to design more specific primers the cDNA sequences for cp-100k were used in conjunction with several *P. pollicipes* ESTs to create a second alignment (Figure 6.9). Identity ranged from 35% (*P. pollicipes* to others) to 70% (between *A. amphitrite* and *F. albicostatus*). Conservation between adhesive protein gene sequences was low; NCBI's BLAST tool had difficulty finding correct matches and the unpublished cp-100k sequences were only found to be similar to *M. rosa* cp-100k using discontiguous megablast (coverage 38-46%, identity/similarity 66-68%).




P. pol:
M. ros:
F. alb:
A. amp:
R2
F2
P3r
polF2


P. pol:
M. ros:
F. alb:
A. amp:
polR2
P7r
R3
F3

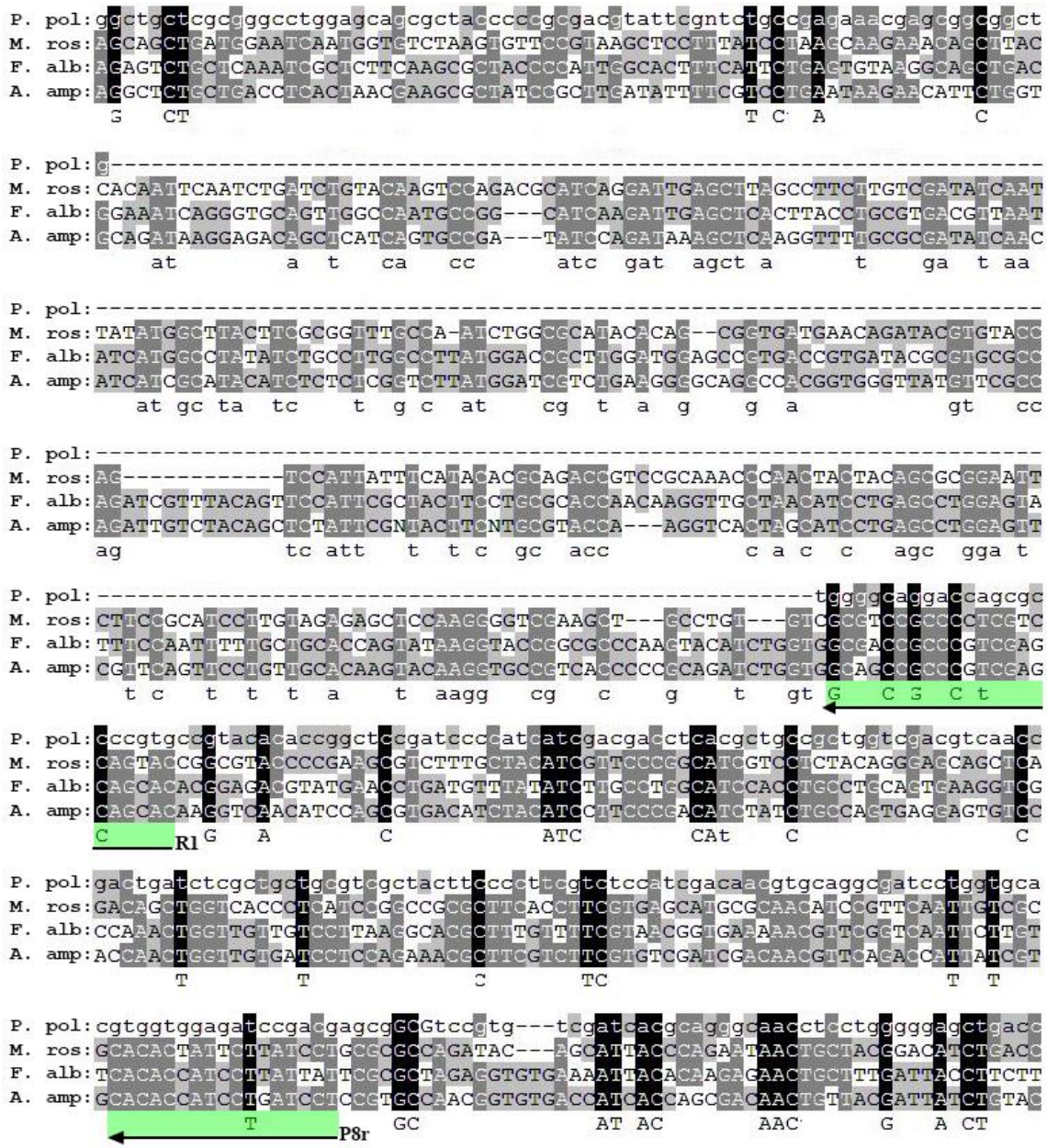


Figure 6.9: Clustal alignment of entire cp-100k genes with sequence conservation shown in shades of grey and primer locations shown in green; arrows indicate primer direction. Dashed lines show base pairs that are not present in a sequence. Consensus sequence is included as the last line of each row.

6.3.3 Amplification of homologues

Overall, attempts to amplify homologous cp-19k, cp-20k and cp-100k adhesive proteins in *Lepas anatifera* using alignments of published sequences met with limited success. Results of PCR reactions were variable, with different amplified products seen when each experiment was repeated. Many of these bands proved difficult to extract from the gel, with very low concentrations of DNA returned. However, on account of the primers being degenerate, sequencing the PCR product directly from the reaction was not possible. The amplified products proved difficult to clone in *E. coli*, with some

reactions resulting in a very low proportion of transformed colonies. These difficulties were an early indicator that the amplified DNA was not the correct product.

Alignments of the amino acid and cDNA sequences of cp-19k from three barnacle species provided suitable sites for three forward and three reverse degenerate primers. All combinations of the six primers were used in PCR reactions containing *L. anatifera* cDNA, with only one combination producing any amplified product. The expected product was approximately 380 base pairs; when the PCR reaction was run through an agarose gel three bands were observed, at approximately 200 bp, 350 bp and 750 bp (Figure 6.10). Repeating the experiment produced the same pattern of bands.

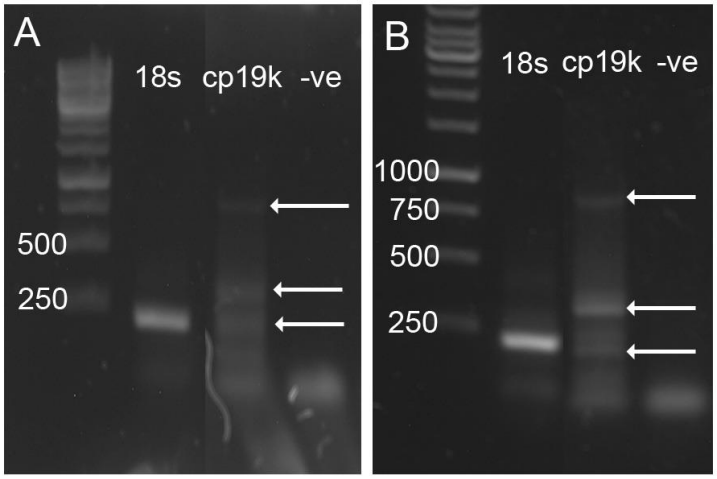


Figure 6.10: Results of two PCR experiments with the cp-19k primer combination F1R2. Distinct bands are present in both experiments at 200, 350 and 750 bp (arrows).

Select bands were cloned and subjected to blue/white screening and PCR screening; clones that were of the correct size (assumed to be the same product) were subsequently sequenced. However, several different sequences were returned. No similarity with the acorn barnacle cp-19k was seen. One sequence contained a conserved peptidase superfamily, with BlastN showing partial matches to ubiquitin-specific peptidases from a variety of species; however the sequence was not an open reading frame, making this hit a false positive. No other sequences showed similarity to anything in the databases, nor did they contain open reading frames that would indicate a protein coding sequence.

Only two published sequences were available for cp-20k and their alignment was poor, thus only two forward and two reverse degenerate primers were produced. Only one of

the primer combinations produced a result. Repeated PCR experiments showed that the products being amplified were not consistent. The first PCR gave a product of approximately the correct size (500 bp) but the second PCR gave three bands, at 200 bp, 300 bp and 500 bp (Figure 6.11).

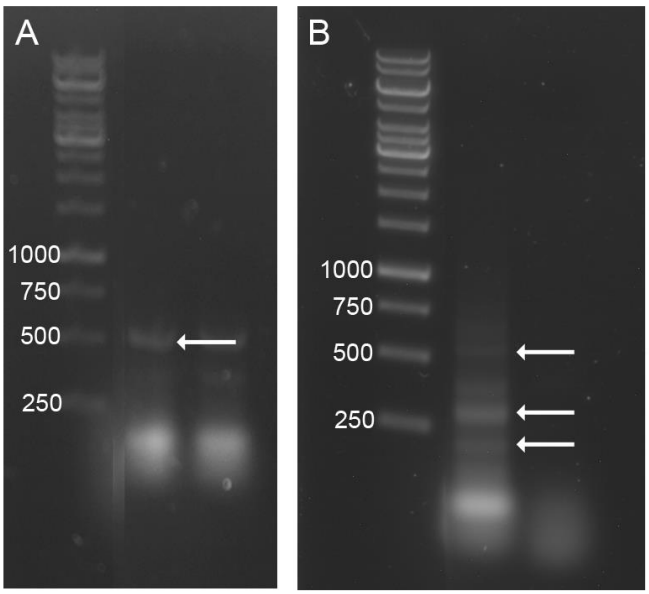


Figure 6.11: Two PCR experiments of *L. anatifera* cDNA with degenerate cp-20k primer combination F1R3: A) a single band was observed at 500 bp; B) three distinct bands were observed at 200, 300 and 500 bp.

Cloning and sequencing of the 500 bp product of the first PCR experiment produced four different sequences. No similarity with the acorn barnacle cp-20k was seen. One sequence showed a partial match to the cytoskeletal protein filamin but overall was non-coding DNA with no open reading frame. Another showed a partial match to the prion superfamily of proteins but also had no open reading frame. The other two showed no homology to anything in the database. It was possible to clone only one of the three products of the second PCR and sequencing produced only one viable sequence. This sequence largely matched the RibA superfamily for GTP-cyclohydrolases.

As can be seen by Figure 6.9, many primer sites were apparent for cp-100k. Degenerate primers were first designed based on three cp-100k sequences, however no significant positive results were found; for several primers a range of products was amplified (Figure 6.12), however the sequences returned contained no open reading frames and most sequences contained only one of the two primer sequences. Blast database searches produced some partial matches but both identity and coverage were low (less

than 50%), indicating that the matches were not actually positive. To increase the chance of a positive result EST sequences were located that matched parts of cp-100k, from which it could be seen that well conserved areas between acorn barnacle species were not conserved in the more distantly related *P. pollicipes*, thus further primers were designed based on an alignment that included *P. pollicipes*.

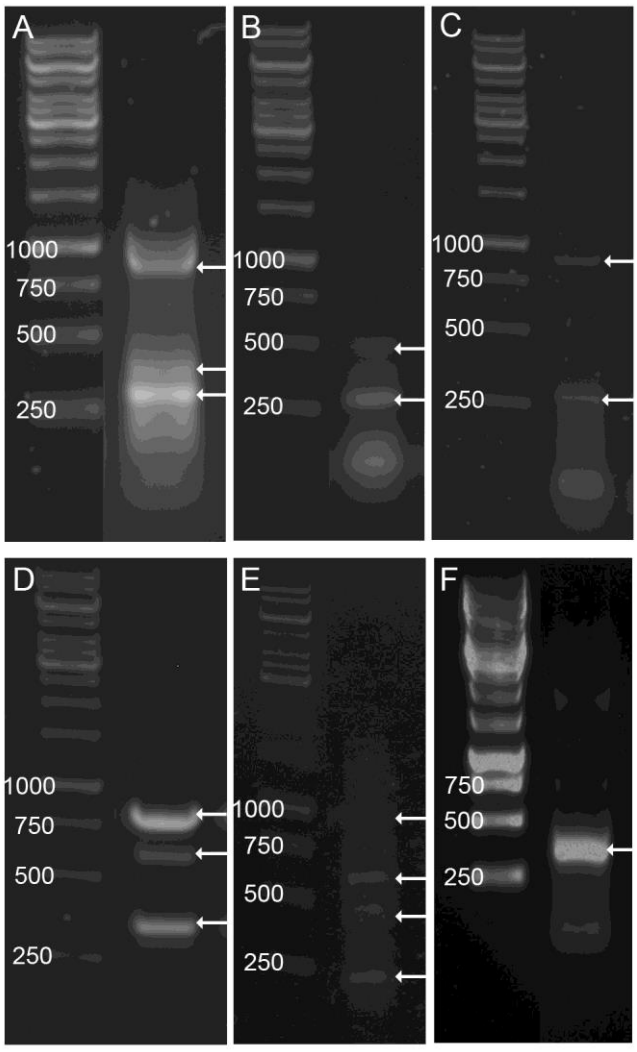


Figure 6.12: Various PCR experiments of *L. anatifera* cDNA with degenerate cp100k primer combinations: A) F3R1; B) F1P3r; C) P7R1; D) F2P7r; E) polF1R1; F) polF2R2.

Including *P. pollicipes* in the alignment did not produce more successful primers. PCR amplified products of varying sizes and results were not consistent when the experiments were repeated. Sequencing the PCR products produced the same results as above, with only non-coding DNA being amplified (no open reading frames, lack of primer sequences, lack of significant database matches). Somewhat interestingly, some PCR experiments amplified products that were similar or exactly the same non-coding

DNA sequences as other PCR experiments using different primer combinations (all based on cp-100k), indicating that despite what appeared to be different results between the two PCR reactions, the same sections of DNA were being amplified. All of the different sequences produced using these primers could be forced into alignment with cp-100k and the *P. pollicipes* ESTs using ClustalW or Muscle, however alignment was very poor, between 29 and 38%, which was reduced further if the primer sequences were removed and only the DNA between the primers was examined.

6.4 Discussion

The results of the current study suggest that homologues of at least two adhesive proteins are conserved between the two barnacle orders examined (Sessilia and Lepadiformes). Immunohistochemistry assays using polyclonal antibodies raised from two *Megabalanus rosa* adhesive proteins (cp-52k and cp-68k) were positive in the adhesive gland cells of *L. anatifera*, as well as in *A. improvisus*. The latter species served as a control due to the fact that cp-68k is apparently conserved between *Megabalanus* and *Amphibalanus* species (Kamino, 2008), as are cp-100k, cp-19k and cp-20k (Kamino, 2008; Mori et al., 2007; Urushida et al., 2007). Localised staining for cp-100k was less convincing; the antibody for cp-100k stained the ovary darkly in both *L. anatifera* and *A. improvisus* and was weaker in the adhesive gland. There was some non-specific staining in ab-cp-52k and ab-cp-68k, as both showed a slight positive reaction in non-adhesive producing tissues. This may be due to the fact that antibodies were polyclonal; a similar observation has been made in an examination of sea-star tube foot adhesion with polyclonal antibodies (Santos et al., 2005). The use of polyclonal antibodies may limit the possible outcomes of immunohistochemistry experiments due to their reduced specificity, however, lower specificity was considered to be a potentially positive factor in the current case, as polyclonal antibodies may counteract minor variations between species and allow a more broad examination across taxonomic groups, as has been carried out in echinoderms (Santos & Flammang, 2012; Santos et al., 2005).

The current results can also be interpreted in the context of recent suggestions of a two-step adhesion process. Burden et al. (2012) concluded that the bulk of the adhesive is not secreted from capillaries in the base plate of acorn barnacles, which have long been assumed to connect with the adhesive gland cells (Lacombe & Liguori, 1969). This implies that there may be a second source of components for barnacle adhesive and that perhaps the adhesive glands produce the second ‘setting’ secretion, with an unknown source producing the first, ‘regular’ secretion. Morphological investigations of the large-bodied stalked barnacles have failed to show any possible protein-secreting cells in the stalk other than the adhesive gland cells. In *L. anatifera* and *D. fascicularis*, the tissue directly adjacent to the adhesive base contained only connective tissue, muscle and epidermis (Jonker et al., 2012; McEvilly, 2011; Zheden et al., 2012).

The evidence in the current study suggests that at least two of the four characterised adhesive proteins are present, as expected, in the gland cells. No evidence was seen in the current study of individual adhesive gland cells secreting different proteins. Each protein appeared to be stained in all cells, although slightly contrasting protein expression patterns were seen. The positive reaction for ab-cp-52k in the adhesive gland cells of *A. improvisus* and *L. anatifera* indicated that this adhesive protein is present and spread homogenously throughout the cytoplasm of each gland cell observed. Cp-68k, on the other hand, appeared to be more densely aggregated around the nucleus of cells. The negative immunohistochemistry result for ab-cp-100k may be a reflection of the highly insoluble nature of this protein. Similar difficulties were experienced when using this antibody for western blotting (K. Kamino 2013, pers. comm. 20 Jan.) and this was attributed to the insolubility of the protein and the possibility that the protein is cross-linked to other proteins or molecules, making the antibody binding sites unavailable. However, a large adhesive protein in a highly folded or cross-linked state within the glandular cytoplasm might be overly obstructive to normal cell functioning. Another explanation of the negative staining results is that of dissimilarity; it has been noted that cp-100k homologues are so dissimilar that after trypsin digest and fragment sequencing, the longest identical tryptic peptide between *M. rosa* and *A. amphitrite* was only four amino acids long (Kamino, 2010b). Further investigations have shown cp-100k to be conserved through several acorn barnacle genera (Kamino, 2008) and results of the present study showed primary sequences to share about 40% identity.

Aligning homologous DNA and protein sequences of the barnacle adhesive proteins cp-19k, cp-20k and cp-100k was only moderately successful; despite the proteins having similar amino acid compositions, the sequence similarity was low. Low conservation of sequences between species was further reflected in our unsuccessful attempts to isolate *L. anatifera* adhesive proteins from degenerate primers based on acorn barnacle adhesive protein sequences. Adding EST sequences from the stalked species *P. pollicipes* to the alignments of cp-19k and cp-100k did not provide more successful primers. Identity between the *P. pollicipes* ESTs and acorn barnacle DNA sequences was relatively low at 26-36%. *Pollicipes* belongs to the order Scalpelliformes and is more closely related and has a similar lifestyle to acorn barnacles than other stalked barnacles such as *Lepas anatifera* (Linse et al., 2013). Thus it is probable that sequence similarity will be lower still between taxonomically distant stalked barnacle species *L.*

anatifera and *P. pollicipes*. The results demonstrate the improbability of discovering functional universal primers to isolate barnacle adhesive proteins from all genera. Isolation of cp-52k and cp-68k via PCR was not attempted as there were no sequences on which to base a primer.

Adhesive proteins have very specific functions, yet in all animal models being studied they do not appear to be highly conserved between genera. The PTM DOPA has long been known to be present in mussel adhesive (Waite & Tanzer, 1981) and its importance for adhesion has been shown through diverse applications of synthetic polymers utilising DOPA chemistry (Lee et al., 2011). DOPA is present in the repeating motif that characterises fp-1, the mussel byssal coating protein and the only byssal protein that has been investigated from a wide range of species, however the repeating sequence varies significantly between genera (Rzepecki et al., 1991). The adhesive of the tubeworm *Phragmatopoma californica* also features DOPA, as well as the PTM pSer, both of which are thought to be important for adhesion (Zhao et al., 2005). Conservation of adhesive proteins in tubeworms is not yet known, however the isolation of some adhesive protein homologues from *Sabellaria alveolata* shows that amino acid sequences are somewhat conserved and that both species utilise repeating sequences in two adhesive proteins and clusters of Ser in a third adhesive protein (Becker et al., 2012). A common feature of mussel, tubeworm and barnacle adhesive proteins is high variation in adhesive proteins between species, which shows that there is great flexibility for these proteins to adapt as required by various environmental factors, yet the function of the proteins remains unaffected. However, while the mussel and tubeworm adhesive proteins share functional characteristics, such as PTMs like DOPA and pSer and the presence of repeating sequences, the barnacle has developed an adhesive strategy that does not utilise these PTMs and which is also lacking repeating sequence motifs.

In the barnacle adhesive, two of the proteins characterised so far are thought to have an adhesive function: cp-19k and cp-20k. The adhesive function of cp-19k has been suggested to rely on the high proportion of Ser, Thr, Lys and Val, which could be useful for coupling to foreign surfaces through hydrogen bonding, electrostatic interactions and hydrophobic interactions (Urushida et al., 2007). The most predictable protein appears to be cp-20k; the primary structure features repeating blocks with Cys residues

at regular intervals and a high proportion of charged amino acids (Mori et al., 2007). The conserved Cys residues have been shown to create a rigid 3-D shape with disulfide bonds (Suzuki et al., 2006) but the latter bonds are shape forming only and have not been suggested to be involved in intermolecular bonding to foreign materials. Two homologous variants of cp-20k have recently been described in *A. amphitrite*; they share a Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Cys motif repeated four times and abundant charged amino acids including His (He et al., 2013). Two other proteins have been characterised as bulk components and not necessarily involved in adhesive function, however the cohesive mechanisms that hold barnacle adhesive together are also a mystery. A pattern of alternating hydrophilic and hydrophobic residues in cp-100k is reminiscent of amyloid proteins and may create a cross-β-sheet structure that forms an insoluble multimer (Kamino et al., 2000). Hydrophobic residues are also present in cp-52k and disulfide bonds are utilised for protein conformation, not cross-linking, thus it has been suggested that hydrophobic interactions are important factors for the cohesion of barnacle adhesive (Kamino et al., 2012).

The amino acid composition and, particularly, the protein conformation may be important factors for the function of barnacle adhesive proteins, but the precise mechanisms used for adhesion and cohesion are unknown. To continue down the path of the current study and isolate adhesive protein homologues from a wide range of barnacle species, from across the entire phylogenetic tree, would allow the researchers to map which areas of each protein are free to change and which remain the same. Beginning with *P. pollicipes* as a link between acorn and stalked barnacles and moving in a stepwise manner through different families of barnacles may finally provide the key to unlocking the mystery that is barnacle adhesion. Highly variable sections of the adhesive proteins may show how the proteins have evolved and been adapted for various different functions while the static, conserved features of the proteins will help researchers to discover what aspects of the proteins are vital for adhesive function.

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7 General discussion

7.1 Research summary

The research outlined in this thesis used a multi-disciplinary approach to examine the adhesive produced by a single species of barnacle, the goose barnacle *Lepas anatifera*. The adhesive production system of the barnacle is unique compared to those of other animals. Strong contrasts between this investigation of *L. anatifera* and reports from other adhesive animal models exemplify that barnacle adhesive is completely distinct from other marine adhesives and does not utilise any of the known underwater adhesive mechanisms. Barnacles have not only developed a form of underwater adhesion that differs from all others that are currently being investigated, they have developed a diverse range of adhesive proteins that are not highly conserved between distantly related barnacle species.

7.2 Adhesive production system

One of the new questions that arose during the course of this study regards the internal adhesive production system, particularly the possibility that there may be a second adhesive component, produced in a separate location and associated with acorn barnacle base-plate growth (Burden et al., 2012). While Burden et al. (2012) provided evidence for a second substance being added to the adhesive plaque at intervals, as of yet no structural information reflects the existence of a second adhesive production area. Zheden et al. (2012) showed that in the stalked barnacle *D. fascicularis* the principal canal that carries that adhesive through the animal branches into many smaller canals as it leaves the animal, similar to the branching capillaries observed in the base plate of acorn barnacles (Burden et al., 2012), however no indication of a second type of secretory cells has been observed in any barnacle species (Jonker et al., 2012; Lacombe, 1970; McEvilly, 2011; Walker, 1970; Zheden et al., 2012). It could be that the same adhesive gland cells produce two different types of adhesive secretion, at different times. It also must be said that, until morphological evidence can be found to support the existence of two adhesive secretions, the second secretion observed by Burden et al. (2012) may be a cuticle secretion and a part of barnacle moulting and growth, not adhesion.

A thorough understanding of the internal structures that produce barnacle adhesive could in turn answer some of the questions surrounding the barnacle adhesive mechanisms, yet it is unlikely that histological and ultrastructural techniques alone will fully resolve this glandular system. To truly understand how and where the adhesive is produced, immunohistochemistry and *in situ* hybridisation will be required to locate adhesive protein gene expression and discover the source of the various adhesive proteins. This may soon become possible for the characterised adhesive proteins cp-19k, cp-20k, cp-52k and cp-100k, however several further adhesive proteins are known to exist and still more unknown components may be present in barnacle adhesive. Investigations of the adhesive production system can only proceed in conjunction with further characterisation of the adhesive proteins.

Further research may lead to the discovery of a second group of cells secreting a further adhesive component, yet the adhesive production system in barnacles will remain incredibly different to what has been observed in other animals, with no distinct granules such as those seen in the body of the tubeworm (Wang et al., 2010) and no multi-gland system like in the mussel foot (Silverman & Roberto, 2010). The barnacle adhesive production system may be two separate single-gland systems, each producing a different adhesive; one that is secreted regularly to make up the bulk of the adhesive and a second that is added in small amounts as the animal grows, is stronger and cures through enzymatic action with a fibrillar, fluorescent, amyloid-like structure.

7.3 Mechanisms of adhesion

The characterisation of the newly discovered second barnacle adhesive secretion as amyloid-like stems from previous studies that have successfully performed Fourier self-deconvolution after FTIR and shown that acorn barnacle adhesive contains a high proportion of amyloid-like cross-β-sheets (Barlow et al., 2010; Barlow et al., 2009; Barlow & Wahl, 2012); in addition, recently the adhesive of the stalked barnacle *D. fascicularis* was tentatively characterised as containing amyloid-like β-sheets, despite the lack of successful Fourier self-deconvolution (Zheden et al., 2013). A pattern of alternating hydrophobic and hydrophilic resides in the primary structure of cp-100k was compared to the alternating polar and non-polar structure of amyloid cross-β-sheets, while secondary structure prediction indicated high presence of β-sheets in cp-100k

(Kamino et al., 2000; Kamino et al., 1996). The presence of amyloid in acorn barnacle adhesive has been shown histochemically (Barlow et al., 2010; Sullan et al., 2009), however histochemical results from the present study showed no amyloid to be present in the adhesive of *L. anatifera*.

There are several possible reasons for these disparate results, the first being that the second, fibrillar adhesive is not always produced (Burden et al., 2012), so may not have been present in the specimens examined in the current study. The formation of amyloid-like fibrils in barnacle adhesive may depend on suitable environmental factors and may be controlled to some extent by salinity and acidity (Kamino, 2008); the slightly acidic pH of the barnacle adhesive gland environment (Jonker et al., 2012) may give the barnacle some control of the formation of cross-β-sheet protein aggregates. It is also possible that the second adhesive secretion was developed after the Lepadiformes, Scalpelliformes and Sessilia orders arose from common ancestor, a division that occurred approximately 252-299 million years ago, during the Permian period (Linse et al., 2013), with Lepadiformes being placed in a basal position (Pérez-Losada et al., 2008). There may be amyloid-like structures in all barnacle adhesive despite the negative results reported here; it has been observed that using the wider interpretation of amyloid to mean fibrillar, extracellular, cross-β-sheet protein aggregates (as opposed to disease causing protein aggregates alone) leads to problems with traditional visualisation methods, as Congo red and Thioflavin T do not always react to all types of amyloid (Fändrich, 2007). Finally, it must be pointed one of the secretions observed by Burden et al. (2012) may yet be shown to be an aspect of moulting or shell growth and not a specialised adhesive secretion. In a bioadhesive the presence of cross-β-sheets, a quaternary protein structure, may promote strength (cohesion) due to sacrificial bonds, however the role that amyloid-like structures may play in adhesion (as opposed to cohesion) remains unknown.

A recent theory that arose from spectroscopic investigations suggested that phenolic compounds may be a factor in barnacle adhesive curing, despite this possibility having been long dismissed (Burden et al., 2012). This supposition was neither ruled out nor supported by the current study. Histochemistry and Raman spectroscopy did not rule out the presence of phenolic compounds in the adhesive gland and adhesive plaque, however the Raman marker for Tyr (a phenolic amino acid) was not strong and any

phenol peak in the *L. anatifera* FTIR spectrum was hidden by the Amide II peak. The utilisation of phenol chemistry by the barnacle cyprid larvae for adhesion has been shown histochemically in the past (Walker, 1971), however it has long been assumed that the cyprid and adult adhesives bear no relation to each other, as the cyprid adhesive gland disappears and is replaced by the adult adhesive gland (Cheung & Nigrelli, 1975; Walley & Rees, 1969). Recent evidence indicates that the cyprid and adult barnacle adhesive may share some features after all, as homologues of the adult adhesive protein cp-20k are present in the cyprid adhesive gland of *Amphibalanus amphitrite* (He et al., 2013). The current results show no convincing evidence that there is a phenolic component in the adhesive, however this aspect of barnacle adhesive clearly needs to be re-examined thoroughly, as enzymatic action could provide the necessary mechanism to explain barnacle adhesive curing.

While spectroscopy techniques have not provided any positive clues for the adhesive mechanisms utilised by barnacles, the results of the current study allow us to rule out some possibilities. There were no strong S–S or S–C peaks, thus any disulfide bonding is likely to be minor and not easily detected; the presence of disulfide bonds are likely, yet have so far only been proven to be present in cp-52k and cp-20k, where they are required for intermolecular shape, not adhesive bonding (Kamino et al., 2012; Suzuki et al., 2006). Phosphorus was not present in *L. anatifera* adhesive and spectroscopic results showed no indication of phosphorylated proteins. No protein-metal interactions were observed in *L. anatifera* adhesive, in contrast to their dominance in the mussel adhesive Raman spectra (Harrington et al., 2010). Finally, comparing the Raman spectra of *L. anatifera* and *Balanus crenatus* showed species-specific differences, reflecting the low homology between species that was seen when attempting to isolate stalked barnacle homologues of acorn barnacle adhesive protein genes.

The post-translational modifications that are vital components of other underwater adhesives are not present in barnacle adhesive, however several proteins remain uncharacterised that could contain post-translational modifications required for adhesive function. Thus far only simple glycosylation has been found in barnacle adhesive proteins (cp-52k (Kamino et al., 2012)), a feature that is likely to be present in other barnacle species and possibly more pronounced in stalked barnacles. Histochemistry revealed the presence of carbohydrates in *L. anatifera* adhesive and adhesive gland,

while a weak, broad FTIR peak at 1080 cm^{-1} was assigned to polysaccharides. In *A. amphitrite* the FTIR polysaccharide peak was only observed in hydrated adhesive, while in *L. anatifera* it was clearly present in dried adhesive and had a similar strength, shape and position to what was observed in *D. fascicularis* dried adhesive (Zheden et al., 2013). Histochemically, reports of carbohydrate in acorn barnacle adhesives are negative (Walker, 1970), although biochemical analyses indicate a very low carbohydrate presence (Walker, 1972), whereas histochemical analyses of the *L. anatifera* adhesive glandular system clearly indicated the presence of carbohydrate in the adhesive.

7.4 Conservation of adhesive components

What has become strikingly clear over the course of the present study is a wide separation between the adhesive produced by stalked barnacle species such as *L. anatifera* and acorn barnacle species. The two clades diverged over 200 million years ago, yet the adhesive proteins have a highly specific function and thus were expected to share enough primary protein structure for homologues of acorn barnacle adhesive proteins to be isolated from *L. anatifera*. While some aspects of the adhesive proteins are likely to be conserved between the two clades, as indicated by positive immunohistochemical results for cp-68k and cp-52k, the low conservation of adhesive proteins raises questions as to the importance of primary protein structure for function. Evidence from barnacle adhesive proteins that have already been well characterised indicate that the proportion of certain amino acids is conserved for some adhesive proteins, such as cp-19k (Urushida et al., 2007), while the shape of the protein is conserved for others, such as cp-20k (Mori et al., 2007; Suzuki et al., 2006).

To fully understand the natural phenomenon that is barnacle adhesion will require the characterisation of the adhesive of many different barnacle species. Currently there is a scarcity of molecular information for barnacles; addressing this knowledge gap is a vital step for the continuation of barnacle adhesion research. The creation and publication of cDNA libraries, genomes and transcriptomes from a wide range of barnacle species will provide a basis for investigating the presence of adhesive protein homologues in all species, including stalked barnacle species. The worth of such methods can be exemplified by a recent transcriptome study that led the researchers to locate

homologues of cp-20k in both adult and cyprid *A. amphitrite* (Chen et al., 2011). The same route has been taken to investigate the adhesive of tubeworms, with the creation of a cDNA library leading to the discovery of many new adhesive proteins (Endrizzi & Stewart, 2009), which were then used to investigate the localisation of adhesive protein expression using *in situ* hybridisation and immunohistochemistry (Wang & Stewart, 2012), leading to new conclusions regarding the mechanisms behind tubeworm adhesive curing and adhesion. The creation of a cDNA library or transcriptome for a staked barnacle such as *L. anatifera* could be used to locate homologues to acorn barnacle adhesive proteins, or more potential adhesive proteins based on characteristics common to many adhesive proteins, as was done by Wang and Stewart (2012). Gathering the same information from a range of species that covered all branches of the barnacle phylogenetic tree could allow researchers to understand the evolution and development of barnacle adhesion and how it has been adapted to many different requirements.

7.5 Conclusions

Barnacle adhesion is in many ways unique compared to other marine animal adhesives. After decades of research only a single secretory cell type has been found to be associated with the adhesive production system, and no separation of the adhesive components has been observed prior to the release of the adhesive to the environment. Barnacle adhesive does not utilise DOPA, phosphorylated proteins or protein-metal interactions. No repetitive sequence motifs have been observed in barnacle adhesive proteins and conservation of adhesive protein genes between genera is low. Homologous adhesive genes in barnacles also have significant differences in pI and size.

The unique adhesive mechanisms developed by barnacles have the potential to inspire many new opportunities for biomimetic applications. While tubeworm and mussel adhesives have already been successfully mimicked to create synthetic adhesives, investigations of barnacle adhesive have not yet progressed so far. One advantage to basing new wet-setting adhesives on barnacle adhesive proteins is that they do not rely on PTMs such as DOPA and pSer for adhesion, allowing functional recombinant proteins to be produced without technical post-production modification and purification

being required. Another advantage is the diversity that exists across barnacle genera, providing the possibility of creating adhesives with different features for various applications and functions. Further investigations of stalked barnacles such as *L. anatifera* will complement the results being gathered from acorn barnacle adhesive and provide further inspiration for researchers that hope to understand underwater adhesion.

7.6 References

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