REARING TEMPERATURE AFFECTS THE EXPRESSION OF PROTEINS IN THE ADHESIVE OF THE STRIPED ACORN BARNACLE, *BALANUS AMPHITRITE*

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Master of Science in Biological Sciences

by

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ABSTRACT

Rearing temperature affects the expression of proteins in the adhesive of the striped acorn barnacle, *Balanus amphitrite*

Melissa J. Daugherty

Barnacles are dominant hard–fouling organisms in marine waters. They attach to substrates by secreting a complex proteinaceous adhesive. Understanding the chemical composition of this multi–protein underwater adhesive and how it is affected by environmental variables, such as oceanic temperatures, is critical for developing nontoxic solutions to control biofouling. Previous experiments in our lab revealed an inverse relationship between critical removal stress (CRS) and temperatures at which barnacles were reared. Further investigations showed that this correlation is not attributed to differences in physical properties such as barnacle size or short–term changes in the viscosity of adhesive. Therefore, the observed effects may be influenced by a physiological response to temperature during initial growth and development. We hypothesized that rearing temperature affects the expression of proteins found in the adhesive matrix. To elucidate the underlying mechanisms responsible for the temperature effect, we analyzed uncured barnacle adhesive using two-dimensional gel electrophoresis (2DGE) and matrix-assisted laser desorption/ionization-tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS). In our analysis, we 1) detected differences in protein expression at two experimental temperatures (15°C and 25°C) and 2) identified several proteins that may serve functional roles in the process of adhesion. Our data are also consistent with a model that the curing process of barnacle adhesive may be analogous to the process of wound healing in animals.

Keywords: Biofouling, antifouling, fouling-release, critical removal stress, proteomics, *Balanus*

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I. INTRODUCTION

Biofouling is the colonization of submerged structures by marine organisms, which can be a two-fold problem with both economical and ecological implications. First, biofouling on the hulls of ships creates drag, lowers fuel economy, and increases greenhouse gas emissions. In addition, it causes corrosive damage, which compromises the structural integrity of ships, often leading to costly repairs (Townsin, 2003; Schultz, 2007; Schultz et al., 2011). This mixed and dynamic community is comprised of a succession of organisms including soft-fouling (marine slimes, bacteria, diatoms, hydroids, algae, etc.) and hard-fouling (barnacles, mussels, tubeworms, bryozoans, etc.) members. Based on calculations from US Naval ships (Schultz et al., 2011), the cost of removal and maintenance related to biofouling can be upwards of billions of dollars annually for the shipping industry alone (extrapolated to both Navy vessels and commercial fleets). Second, historical records show that biofouling on ships is a vector for the global transportation and introduction of non-indigenous and invasive marine organisms (Bax et al., 2003; Davidson et al., 2009). These alien species may adversely impact the biodiversity of local marine communities (Bax et al., 2003). For example, San Francisco Bay, California suffers from having virtually every coastal habitat dominated by one or more non-indigenous species (Bax et al., 2003). The worldwide combination of financial and environmental burdens imposed by fouling species in marine systems has encouraged a series of scientific inquiries on the topic of biofouling remediation (for review see Callow & Callow, 2011; Cao et al., 2011).

Historically, the mitigation strategies mainly consisted of antifouling bottom paints and coatings. Although many of these coatings reduced biofouling, the addition of toxic compounds used to make them can be detrimental to non-target marine organisms (Bellas, 2006, 2007). In fact, the ecotoxicological problems associated with leaching of these highly toxic compounds resulted in legislation (IMO Resolution A. 895 21, 25/11/1999) banning the application of organotin-based (carbon and tin) coatings on ships (Bellas, 2006, 2007). In response to this legislation, there is an increased effort to develop environmentally benign and fouling-release coatings as a nontoxic alternative (Bellas, 2006, 2007; Wendt et al., 2006). The goals of these

coatings are to assure continued efficient operation of ships while protecting the environment (Holm et al., 2006). The advancement of biofouling research is producing improved antifouling and fouling-release materials. However, information about the interactions between coating performance and adhesive abilities of fouling organisms under various environmental parameters is limited.

Barnacles are common marine fouling organisms and thus serve as good study organisms for research investigating biofouling on submerged structures. The striped acorn barnacle, *Balanus amphitrite* (Darwin, 1854) is a common species of barnacle utilized in laboratory assays in part because it is easy to culture, fast growing, and has a circumglobal distribution (Callow et al., 2007). Barnacles attach to substrates by secreting a proteinaceous cement compound (Kamino, 1996, 2001, 2006, 2008; Khandeparker & Anil, 2007; Nakano et al., 2007; Naldrett & Kaplan, 1997; Urshida et al., 2007; Walker, 1971). Understanding the composition and biochemical properties of this underwater adhesive is relevant to biofouling research and will aid in developing nontoxic coatings.

Despite the importance of this topic, the molecular mechanisms and specific molecules responsible for attachment have not been fully elucidated (Thyiyagarjan & Qian, 2008; Aldred et al., 2013). Moreover, the environmental variables and oceanic conditions influencing the adhesive properties of these substances are poorly understood. Once permanently attached, barnacles and other fouling organisms are exposed to a broad range of environmental conditions, including variable sea surface temperatures, as the ships they are attached to move through drastically different oceanic zones (eg polar regions to tropical oceans). For example, a ship traveling along the temperate coast of California (from the port of San Diego to Humboldt Bay) will experience an average sea surface temperature change of 10°C (www.ndbc.noaa.gov) over the course of approximately 645 nautical miles and 2.7 days (www.sea-distances.org). This example illustrates the need for determining the effects of temperature fluctuations on adhesive properties of these animals. Moreover, this information would be extremely useful in informing biofouling research on the performance of environmentally benign fouling-release coatings.

Experiments investigating the influence of environmental conditions on the critical removal stress (CRS-the amount of force normalized to area required to dislodge a barnacle from a substrate) for barnacles reared at different temperatures, and on different coatings, have demonstrated a notable trend. Barnacles grown at a lower temperature required a greater removal force compared to those grown at higher temperatures (Johnston, 2010). To ensure these observations were not attributed to barnacle size (animals grown at colder temperatures are typically smaller than those grown in warmer temperatures), CRS was plotted against basal plate diameter. These data showed no significant correlative effect of removal force due to size (Johnston, 2010). This suggests the adhesive properties of barnacle cement itself can be affected by temperature changes. These observations could be attributed to: 1) physical mechanisms: for example, changes in viscosity of the adhesive, or, 2) biological mechanisms: for example, organismal regulation of adhesive quality.

Follow-up studies investigated the potential physical changes in viscosity of the adhesive due to removal temperature (colder temperature potentially having more viscous adhesive compared to warmer temperature). Two groups of barnacles were reared at 15°C and 25°C respectively for approximately three months to reach an appropriate experimental size (~5mm base plate diameter). At that time, half of the barnacles from the 15°C reared group were switched into 25°C, and half of the barnacles from the 25°C reared group were switched into15°C. Both groups were allowed to acclimate to the new temperatures over a few hours, prior to taking removal force measurements. Results from this temperature swap experiment showed no correlation between CRS and removal temperature; however, there was a significant effect of rearing temperature (**Fig. 1**, Wendt, unpublished).

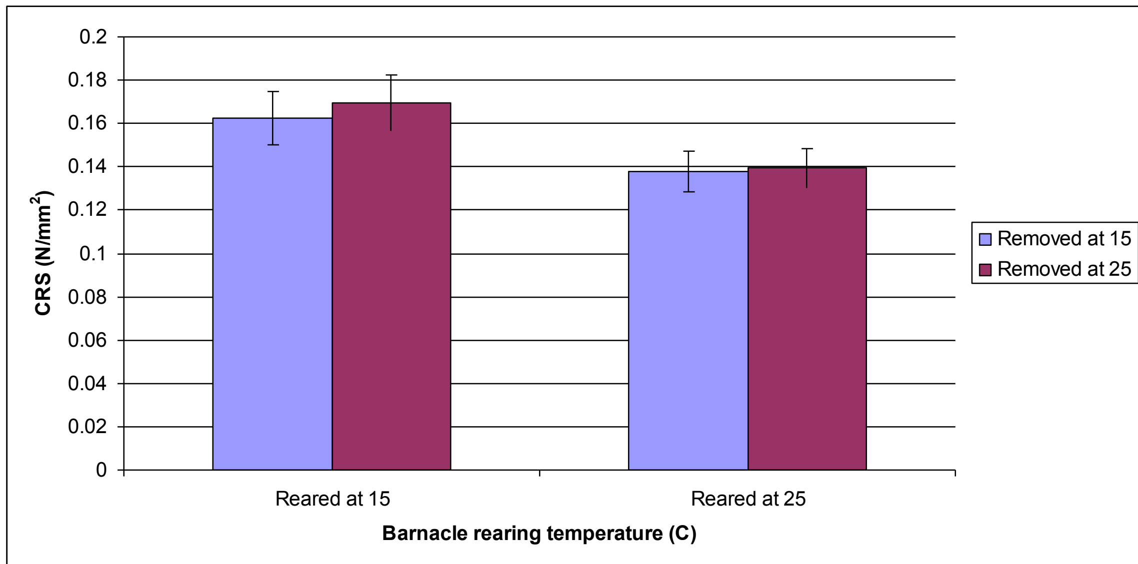

Figure 1. Average critical removal stress (CRS) for *Balanus amphitrite* reared and removed at two different temperatures. No significant difference in CRS was determined for removal temperature after barnacles were swapped into opposite incubators and removed post acclimation to the alternative temperatures (2-way ANOVA, $p = 0.6705$). However, a significant difference in CRS was demonstrated for barnacles based on original rearing temperatures (15°C and 25°C, respectively) (2-way ANOVA, $p = 0.0142$).

This experiment showed a consistent inverse relationship between CRS and rearing temperature previously demonstrated by Johnston (2010) where the 15°C reared group experienced significantly higher CRS at both removal temperatures compared to the 25°C reared group (**Fig. 1,** Wendt, unpublished). These results suggest that rearing temperature and not removal temperature is an important underlying factor. Additionally, there appears to be a long-term, physiological component that influences the tenacity or mechanism of adhesion that cannot simply be explained by acute changes in ambient water temperatures. One way to address this physiological aspect is to use a two-dimensional proteomic analysis to test for variations in composition of the proteinaceous adhesive when barnacles are reared at different temperatures. An investigative experiment following Johnston's original study provided preliminary evidence that

these types of changes in barnacle adhesive proteins can be detected using this approach (Johnston, 2010).

Proteomics identifies and characterizes the global protein expression within a biological sample. It is a tool used to infer function based on the identification of individual proteins and to catalog proteomes (Wilkins et al., 1996). Most importantly, it is a method used to monitor changes in the overall protein expression in various environmental or experimental conditions (Görg et al., 2004; Thiyagarajan & Qian, 2008). Although sequenced genomic information for barnacle species is incomplete, the potential application of a proteomic study to investigate *B. amphitrite* adhesive proteins is promising. (For reviews of proteomic studies using non-model organisms, see Tomanek 2006, 2014). Adhesive proteins termed "cement proteins" have been identified in a number of barnacle species, including *B. amphitrite* (see Kamino, 2008, 2010, and 2012 for review) and additional studies have also generated expressed sequence tags (EST) libraries (Bacchetti De Gregoris et al., 2009; Bacchetti De Gregoris et al., 2011) that can be used for comparison. Interestingly, other studies have proposed a model of barnacle biomolecular adhesion based on the evolutionary concepts of wound healing and have described some proteins that could be homologous to those involved in blood clotting activities (Dickinson et al., 2009). These previously characterized proteins and libraries will provide the basis for a targeted approach to identify additional proteins of interest in *B. amphitrite* adhesive.

My study focused on the collection of uncured adhesive and utilized a proteomic strategy to identify and characterize barnacle adhesive proteins. I expected to: 1) detect changes in protein expression due to rearing temperature, and 2) identify proteins and infer their potential involvement in adhesion. This proteomic approach sought to elucidate the effects of temperature on adhesive composition and functional abilities of this ubiquitous fouling species. Results from this study would be instrumental in increasing our understanding of underwater attachment of marine fouling species, and will provide knowledge of an unique adhesive system that could improve antifouling/fouling–release technologies aimed at alleviating the impacts of biofouling.

II. MATERIALS AND METHODS

Animal Collection and Maintenance

Balanus amphitrite cyprid larvae were allowed to settle on fouling–release coatings (Dow Corning Silastic® T2 PDMS elastomer) for easy removal. A drop assay (placing drops of seawater containing 20–40 larvae onto the face of a slide) was utilized for settlement (Callow et al., 2007). The larvae were allowed to settle for 72 hours in covered petri dishes at 25°C. Slides containing newly metamorphosed juveniles were placed into individual petri dishes and filled with a mixed phytoplankton culture (15mL each of *Dunaliella sp.* and *Skeletonema costatum*). The dishes where then haphazardly divided and transferred to assigned temperature incubators (15°C and 25°C respectively) set on 12h light/dark cycle. Barnacles from each treatment were fed identical diets (consisting of fresh cultures of the mixture described above) three days a week. During feedings, old culture was removed, and slides were observed for overcrowding and gently wiped clean of any algal growth. This feeding and cleaning schedule continued for approximately one month before barnacles were switched to zooplankton diets consisting of 1ml concentrated *Artemia spp.* nauplii larvae suspended in 40ml sterile filtered seawater (see Wendt et al., 2006 for review of methods). Barnacles were again fed identical diets three times a week (with fresh *Artemia spp.* cultural), and slides were maintained clean of any residual algal growth until they reached a sufficient experimental size of at least 5mm baseplate diameter (approximately three months).

Experimental Design

Methods utilized for collecting uncured barnacle adhesive were adapted from Dickinson et al., 2009. Adult barnacles were removed from slides, wiped clean with kimwipes and sterile deionized water, and subjected to mechanical probing (using a sterile dissection needle) at the basal plate/lateral plate interface to initiate secretion of uncured, liquid adhesive (Dickinson et al., 2009). The adhesive droplets were collected using a micropipette and stored in a sterile epenndorf tube containing sample buffer.

Uncured adhesive samples were randomly collected from multiple individuals and suspended in rehydration buffer [7M urea, 2M thiourea, 2% cholamidopropyl–dimehtylammonio–propanesulfonic acid (CHAPS), 2% nonyl–phenosylpolyethoxylethanol–40 (NP–40), 0.002% bromophenol blue, 0.5% ampholyte and 100mM dithioerythreitol (DTE)] at a ratio of 1µl of adhesive to 20µl buffer. These pooled protein samples were vortexed to ensure complete mixing and then stored at –80°C. Protein concentrations of each sample were later quantified using the 2D Quant Kit (GE Healthcare) according to the manufacturer's instructions.

Two–Dimensional Gel Electrophoresis (2DGE)

For each pooled adhesive sample, a total of 100µg protein was added to 11cm, pH 3-10 non-linear immobilized pH gradient gel strips (ReadyStrip IPG strips - BioRad, Hercules, CA, USA) and allowed to passively rehydrate for up to 12 hours. The rehydrated gel strips were then run on an IPGphor 3 (GE Healthcare) isoelectric focusing system with the following parameters: 500 V (rapid increase, 0:15 hr). 8000 V (gradient, 1:00 hr), 8000 V (steady, 3:45 hr) and 500 V (hold). Gel strips were stored frozen at -80°C.

Gel strips were placed in equilibration buffer (375mM tris–base, 6M urea, 30% glycerol, 2% sodium dodecylsulfate (SDS), 0.002% bromophenol blue) mixed with 10mg ml^{-1} DTT and incubated on an orbital shaker for 15 minutes. Next, gel strips were incubated with equilibration buffer mixed with 135mM iodoacetamide and placed on an orbital shaker for an additional 15 minutes. The gel strips were then seated on top of 12% SDS–polyacrylamide gels and bound in place with 0.8% agarose solution containing Laemmli SDS electrophoresis running buffer (25mM tris–base, 192mM glycine, 0.1% SDS).

Gels were placed in a Criterion Dodeca electrophoresis unit (BioRad) with a recirculating water bath at 12°C and run at 200 V for 55 minutes. Gels were incubated twice in a fixing solution (10% methanol, 7% glacial acetic acid) for 30 minutes each. Gels were rinsed in MiliQ and stained overnight with SYPRO Ruby florescent stain (BioRad) in the dark following manufacture recommendations (BioRad). The following day, gels were destained using the same protocols for the fixing solutions (incubated twice in 10% methanol, 7% glacial acetic acid for 30

minutes each). Gel images were captured via scans completed by a typhoon Trio+ Flourescent imager (GE Healthcare).

Gel Image Analysis

Scanned gel images were analyzed using the software package Delta2D (version 3.6; Decodon, Greifswald, Germany). The digital gel images were manually edited and merged together using the match vector approach in the program's group warping strategy. This function creates a fused composite image, or proteome map, of all the gel images representing the average spot volumes of each detected spot. This composite image was further edited to determine spot boundaries that were then transferred back onto each respective gel image via the linked match vectors. Background "noise" was then deleted from the image as a clean up step before normalizing the protein spot volume against the total spot volume of all the proteins within a gel image.

Statistical Analysis

Delta 2D was used to analyze normalized spot volume with a Student's T test to detect differences between the two temperature treatments (15°C and 25°C, respectively). A null distribution was generated using 1,000 permutations to account for unequal variance and non–normal distributions of the protein spots, setting a stringent alpha level of 0.02 to be more conservative and to reduce the likelihood of false positives. Hierarchical protein clusters were generated in Delta2D using average linking with Pearson's correlation metric.

Mass Spectrometry– 2DGE MALDI-TOF/TOF

Proteins that changed in abundance due to temperature treatment, and those that fell within the molecular ranges of previously identified barnacle adhesive proteins, were excised from gels using a BioRad ProteomeWorks automated spot picker (in fluorescent mode). Destaining buffer (25mM ammonium bicarbonate in 50% acetonitrile) was added twice (30 minute incubation each on a rotating shaker) to remove SYPRO stain from the gel plugs. Plugs were then dehydrated using 100% acentonitrile, and proteins were digested overnight in 11ng μl^{-1} trypsin

solution (Promega, Madison, WI, USA) at 37°C. Digested peptides were eluted twice from the gel plugs using an extraction buffer (0.1% trifluoroacetic acid (TFA)/acetonitrile; 2:1), and the resulting samples were centrifuged until they were completely dehydrated using a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA). The concentrated peptides were rehydrated by adding 1µl of extraction buffer and then mixed with 5µl matrix solution (0.2mg ml^{-1} α–hydroxycyano cinnamic acid in acetonitrile) and spot plated onto a metal Anchorchip™ target (Bruker Daltonics Inc., Billerica, MA, USA). The peptide spots were washed with a 0.1% TFA solution followed by a recrystallization step using an acetone/ethanol/0.1% TFA (6:3:1) solution.

Mass spectrometry was performed using a Matrix Assisted Laser Desorption/Ionization Tandem Time of Flight Mass Spectrometer (MALDI-TOF/TOF MS, Ultraflex II, Bruker Daltonics Inc.). Peptide mass fingerprints (PMF) were generated for each sample spot, and tandem mass spectrometry (MS/MS) was carried out on the 12 most intense peaks obtained from the original mass spectrum (MS) for each sample. These spectra were processed and analyzed using flexAnalysis (version 3.0; Bruker Daltonics Inc.) with the TopHat algorithm for baseline subtraction, the Savitzky–Golay analysis for smoothing (0.2 m/z; number of cycles=1), and the SNAP algorithm for peak detection (signal–to–noise ratio: 6 for MS and 1.5 for MS/MS). The assumed peptide charge state was +1, and porcine trypsin was used for internal mass calibration.

Protein identification searches were performed using Mascot software (version 3.1; Matrix Science Inc., Boston, MA, USA) with PMFs and tandem mass spectra combined to search against multiple in-house maintained databases [downloaded from the National Center for Biotechnology Information (NCBI) website and updated various times] including several taxonomic classification levels including: Genus = *Balanus*, Subphylum = Crustecea, Phylum = Arthropoda, as well as other invertebrate libraries (eg *Petrolisthes*, *Mytilus*, and *Daphnia*). Additional fasta files for *B. amphitrite* EST libraries were obtained from the Sequence Analysis and Management System SAMS-2.0 for project *SAMS_MGE_amphitrite_2010* (Bacchetti De Gregoris et al., 2011). Variable modifications within the search included oxidation of methionine, carbamidomethylation of cysteine and acetylation of lysine. Minimum criteria were set to accept

one missed trypsin digestion cleavage, a mass peptide tolerance of 0.25 Da and MS/MS tolerance of 0.6 Da. Searches were conducted using the molecular weight search (MOWSE) method where the protein identification was deemed significant if the MOWSE score was higher than a certain threshold value (database dependent) at the $p < 0.05$ level.

III. RESULTS

Significantly Differently Expressed Proteins Between Temperature Treatments

Using a proteome map generated from all gels in the experiment, a total of 291 spots were detected. Of these, 20% (57 spots) were determined to show significant changes in protein abundance across the treatment groups (Student's T test, $p<0.02$. **Fig. 2**).

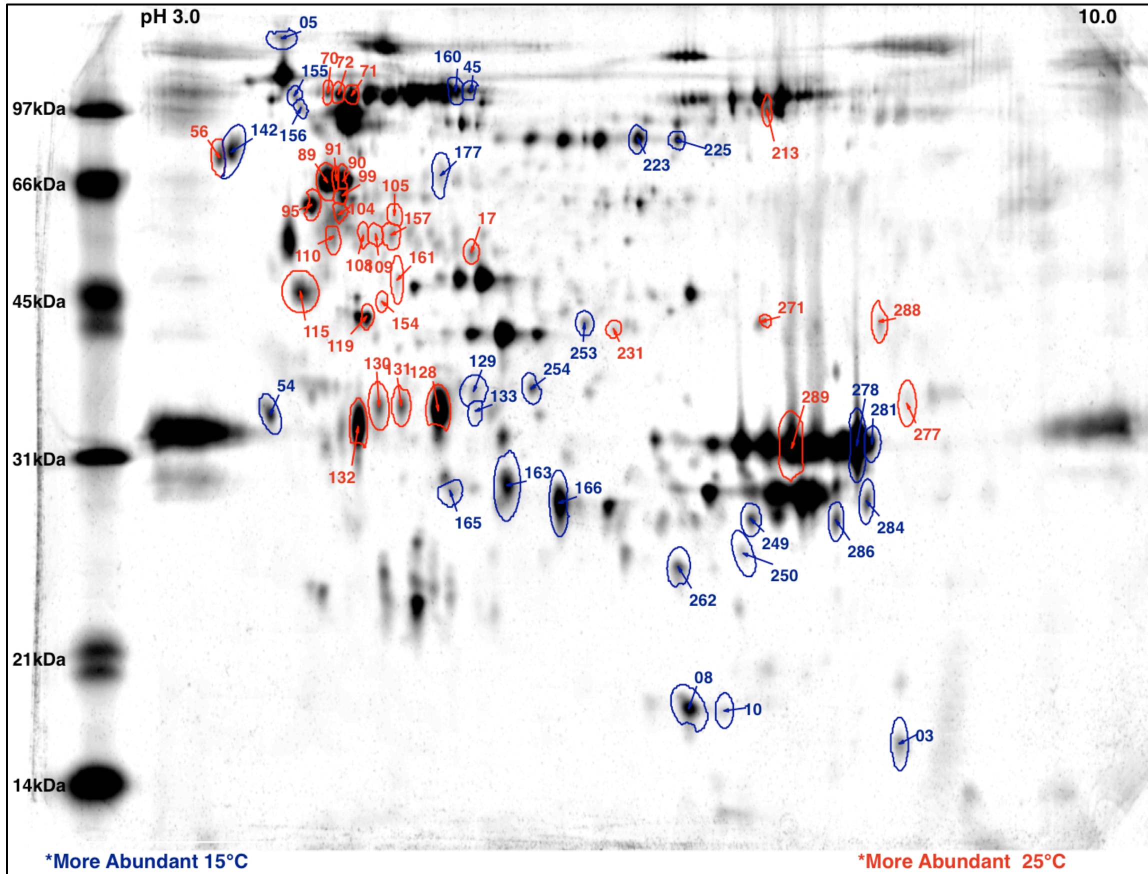

Figure 2. A composite gel image, or proteome map of uncured *Balanus amphitrite* adhesive. Two hundred ninety-one (291) individual protein spots were detected. The proteome map represents the average normalized pixel volumes for each protein spot across 20 sample gels. Each sample gel was generated from separately pooled adhesive samples collected from multiple individuals from each treatment group (15°C and 25°C, respectively). Numbered spots indicate those that showed significant changes in abundance between the two temperature treatments (Student's T test, p<0.02). Red circled proteins were more abundant at 25°C, and blue circled proteins were more abundant at 15°C.

Hierarchical Clustering of Significant Proteins Between Temperature Treatments

Hierarchical clustering grouped the 57 significantly different protein spots into two clusters containing proteins that display similar patterns of protein expression both within and between the two experimental temperature groups. The first cluster contains 27 protein spots that showed significantly greater expression at 15°C, where the second cluster contains 30 protein spots that showed significantly higher expression at 25°C (**Fig. 3**).

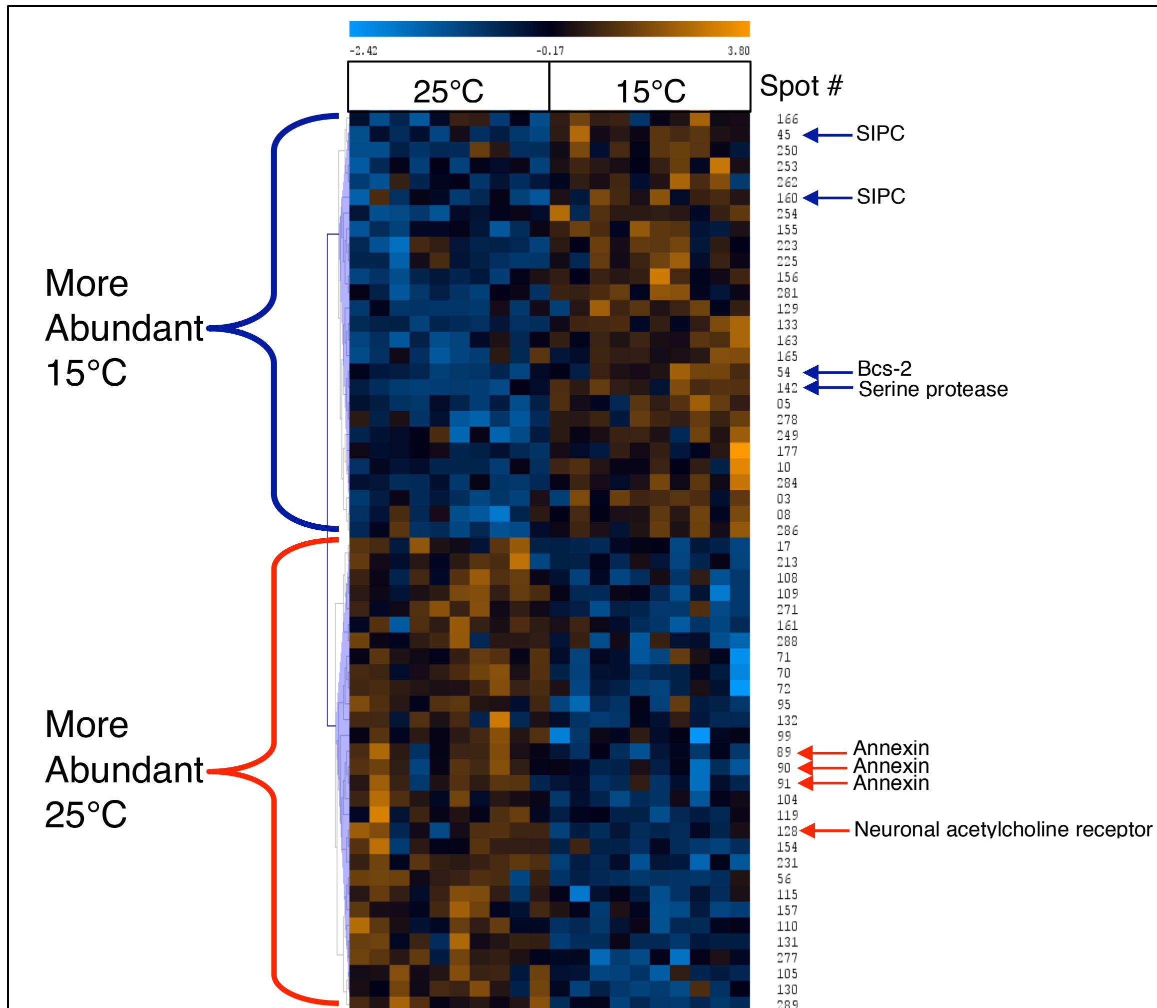

Figure 3. Hierarchical clustering of differentially expressed *Balanus amphitrite* adhesive proteins in response to temperature by Pearson's correlation. Color scale across the top represents proteins ranging from lower than average standardized spot volume in blue, to greater than average standardized spot volume in orange. Temperature treatments are on the horizontal axis (10 columns on left are 25°C, 10 columns on the right are 15°C). Numbers to the right side of the figure are arbitrarily assigned to each protein spot during the initial analysis. Each square represents a single unique protein spot on a single gel. Each column represents all the significant proteins from within a single gel, whereas each row represents a single significant protein among each of the gels. The clustering shown along the left vertical axis shows two major groups of proteins with similar patterns of abundance.

Identification of Significant Proteins Between Temperature Treatments

Protein identification relied heavily on comparing peptide sequence information generated from the various mass spectrometry results, to match against published databases to determine homology or similarity to known proteins. Five proteins (represented by eight spots, or approximately 14% of the total significant spots) of the 57 protein spots that were expressed significantly differently between the two temperature groups were positively identified using MALDI-TOF/TOF MS analysis (**Fig. 4, Table 1**).

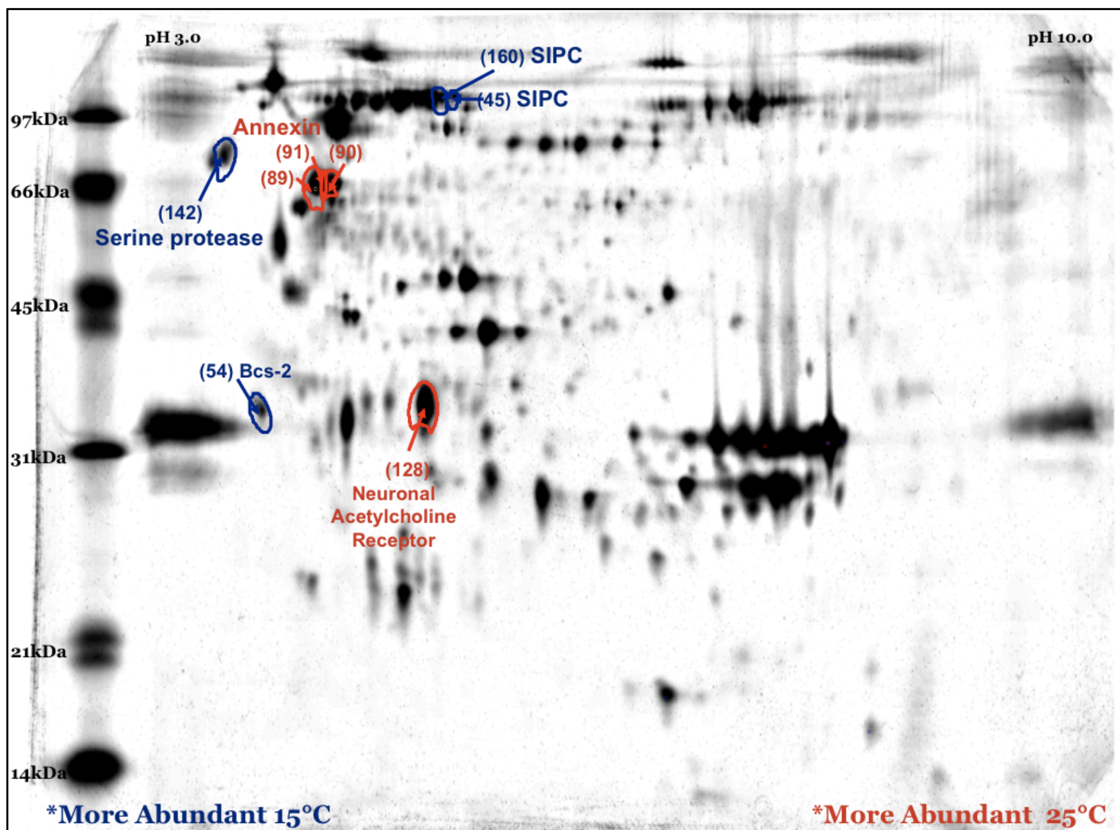

Figure 4. Identified significant protein spots in uncured *Balanus amphitrite* adhesive. Five unique proteins were identified by MALDI-TOF/TOF MS analysis. Three of the identified proteins were more abundant in the 15°C treatment group (blue), while the remaining two were more abundant in the 25°C treatment group (red).

Table 1. Adhesive protein identifications (using MS/MS) of spots significantly changing abundance due to rearing temperature of *Balanus amphitrite.* Sequence Analysis and Management System (SAMs) Identifier represents EST sequence assembly categories derived from the SAMS-2.0 summary for project SAMS_MGE_amphitrite_2010 (Bacchetti De Gregoris et al., 2011). Mascot Score and Sequence Coverage are reflective of these EST sequences. Protein identifications are based on NCBI BLAST results of these translated sequences. Theoretical pI and MM derived from ExPASy Bioinformatics Resource Portal.

Spot ID	SAMs Identifier	Protein ID (NCBI Blast)	pI Theoretical	MM Theoretical (kDa)	GenBank ID	Mascot Score	Sequence Coverage (%)	Proposed Functions
89	Adult_Isotig_isotig01507	(predicted) Annexin	6.02	55.0	GI:242003608	98	13	calcium-dependent phospholipid binding, calcium ion binding
90	Adult_Isotig_isotig01507	(predicted) Annexin	6.02	55.0	GI:242003608	52	7	calcium-dependent phospholipid binding, calcium ion binding
91	Adult_Isotig_isotig01507	(predicted) Annexin	6.02	55.0	GI:242003608	163	14	calcium-dependent phospholipid binding, calcium ion binding
54	NA - matched NCBI Database for Metazoa	Bcs-2	4.22	27.7	GI:9186886	78	16	putative heme-binding proteins
128	Cyprids_Isotig_isotig05664	(predicted) Neuronal Acetylcholine Receptor	6.66	47.9	GI:321458305	66	29	transport, membrane, extracellular ligand gated ion channel activity, neurotransmitter-gated ion-channel ligand binding domain
142	BAMP_Isotig_isotig_01024	(predicted) Serine Protease	5.22	39.8	GI:157130423	161	25	Secreted trypsin-like serine protease [posttranslational modification, protein turnover, chaperones]
45	Cyprids_Isotig_istotig00848	Settlement Inducing Protein Complex	4.95	170.7	GI:71361896	52	5	endopeptidase inhibitor activity, extracellular space, alpha-2 macroglobulin
160	Cyprids_Isotig_istotig00848	Settlement Inducing Protein Complex	4.95	170.7	GI:71361896	236	7	endopeptidase inhibitor activity, extracellular space, alpha-2 macroglobulin

Relative Abundance of Proteins Expressed Significantly Higher at 15°C

The three proteins that were determined to be more abundant in the 15°C treatment group include a barnacle cyprid specific protein (Bcs-2), a settlement inducing protein complex (SIPC), and a serine protease (**Fig. 5**).

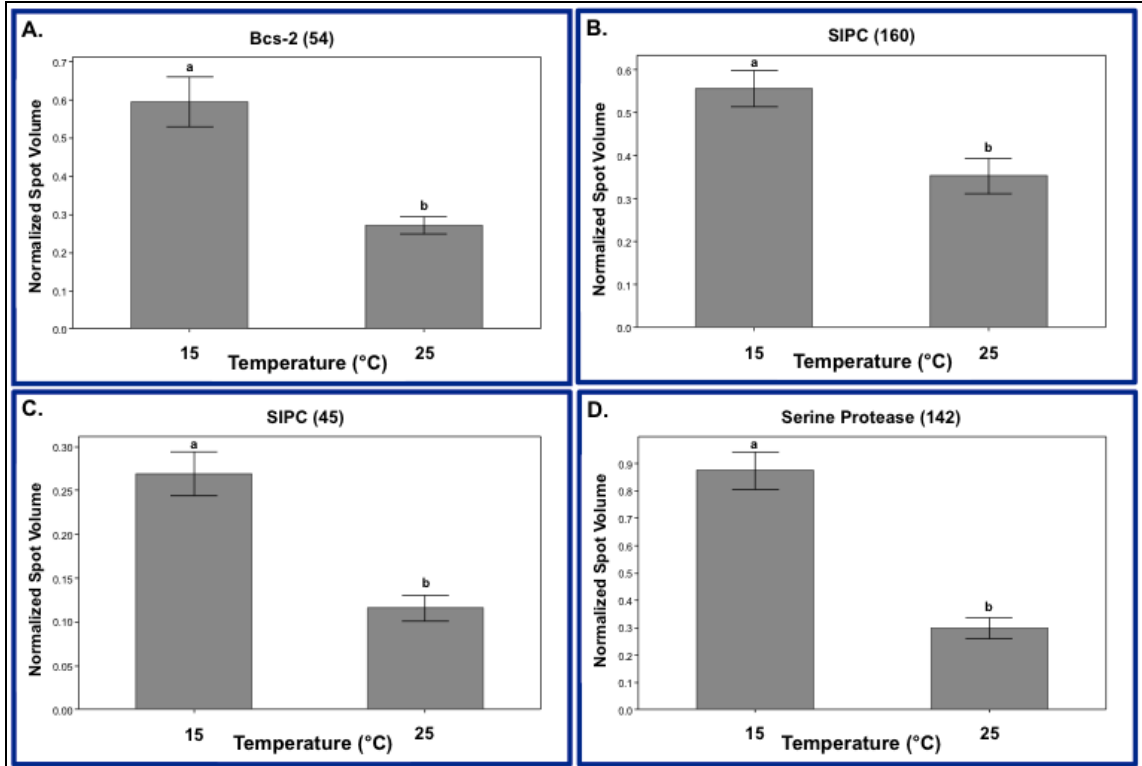

Figure 5. Bar graphs showing the relative abundance of proteins expressed significantly higher at 15°C. (A) Barnacle cyprid specific protein 2 (Bcs-2 - spot no. 54), (B, C) Settlement Inducing Protein Complex (SIPC – spot nos. 160 & 45), and (D) Serine protease (spot no. 142). Spot volumes were obtained by normalizing against the volume of all proteins, and means ± 1 s.e.m. are shown (N=10 for all groups). Letters above bars indicate significant differences in protein abundance level between the two temperature treatments).

Bcs-2 (spot no. 54) showed over two times the increased level of protein expression at 15°C compared to 25°C (**Fig. 5, A**). SIPC showed approximately one and a half times increased level of protein expression (spot no. 160), and over two times the increased level of protein expression (spot no. 45) at 15°C compared 25°C (**Fig. 5, B & C**). Serine protease (spot no. 142) showed nearly three times the increased level of protein expression at 15°C compared to 25°C (**Fig. 5, D**).

Relative Abundance of Proteins Expressed Significantly Higher at 25°C

The two proteins that were determined to be more abundant in the 25°C treatment group include a class of neurotransmitter receptors (acetylcholine receptor protein) and a member of the functionally diverse annexin protein family (**Fig. 6**).

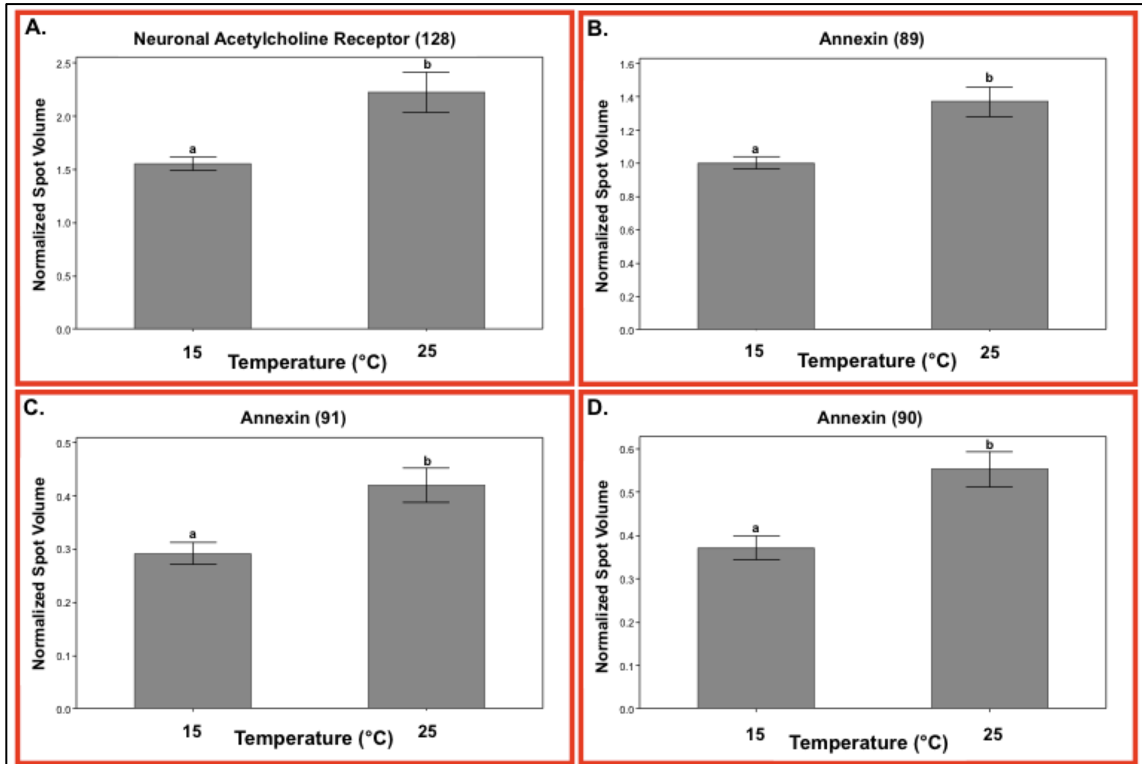

Figure 6. Bar graphs showing the relative abundance of proteins expressed significantly higher at 25°C. (A) Neuronal acetylcholine receptor (spot no. 128), (B-D) Annexin (spot nos. 89, 91, & 90). Spot volumes were obtained by normalizing against the volume of all proteins, and means ± 1 s.e.m. are shown (N=10 for all groups). Letters above bars indicate significant differences in protein abundance level between the two temperature treatments.

Neuronal acetylcholine receptor protein (spot no. 128) showed nearly one and a half times the increased level of protein expression at 25°C compared to 15°C (**Fig. 6, A**). Annexin (spot nos. 89, 91, & 90) showed approximately one and a half times the increased level of protein expression at 25°C compared 15°C (**Fig. 6, B-D**).

IV. DISCUSSION

The cosmopolitan distribution of *Balanus* barnacles, in addition to the ability to grow them in the lab and the legacy of cement research, makes them a relevant model organism for biofouling research. To date there have been many studies aimed specifically at identifying the adhesives produced by this common fouling species and in understanding how these adhesives function to adhere the organisms to substrates (Kamino, 2013). Though there has been a great deal of progress in the research with respect to the attachment mechanism, there is still much to learn about the biochemical and physiological components and processes of adhesion.

Our study was conducted to explore the physiological mechanisms responsible for the observed inverse relationship in adhesion strength between barnacles reared at two biologically relevant temperatures (15°C and 25°C) (Johnston, 2010). Knowing that barnacle adhesive is highly proteinaceous with a number of known barnacle 'cement proteins' (Kamino, 2013), and that changes in protein abundance in uncured barnacle adhesive could be detected (Johnston, 2010), our study examined differences in the expression of these (and other) proteins that could be responsible for the effect of temperature on removal force. We determined that the protein composition (type and abundance) of uncured *B. amphitrite* adhesive varied for barnacles raised under two different temperature treatments by detecting a significant change in the abundance of 57 of 291 protein spots between the two groups (15°C and 25°C, respectively). These results provide supporting evidence that changes in protein composition could contribute to observed differences in adhesive tenacity previously described (Johnston, 2010).

Proteins Expressed in Higher Abundance in the 15°C Treatment Group

Bcs-2 – This gene was first described as one of six cDNAs thought to be expressed specifically during the barnacle cypris stage (bcs) of larval barnacle development (Okazaki et al., 2000). The study showed mRNA expression of this particular gene to be between 0 and 7 hours with decreased mRNA activity toward the progression of attachment and metamorphosis; thus, bcs-2 was designated as an early stage cyprid gene (Okazaki et al., 2000). The full-length nucleotide sequence of bcs-2 (1259bp, NCBI Accession No. AB021903) corresponded to a polypeptide of

246aa (~26.7kDa, pI 4.06) that showed no significant nucleotide or amino acid sequence similarities with other databases (Okazaki et al., 2000). Another study investigating the expression level of these genes during cyprid attachment and metamorphosis found the same trend in decreased expression levels of bcs-2 during metamorphosis (Li et al., 2010). The authors of this study also reported sequence homology related to a putative heme-binding protein, though provided no discussion about functional significance (Li et al., 2010).

While some bcs genes appear to be uniquely expressed during the cyprid stage (notably, bcs-1, -3 and -4), bcs-2 transcripts have been detected in both naupliar and cyprid ESTs, suggesting that Bcs-2 is not necessarily specific to one stage of development (Bacchetti De Gregoris et al., 2011). Though genes expressed in each developmental stage are likely to be highly diverse, cyprid- and naupliar-specific genes appear to be more similar to each other than to the adult gene assemblies (Bacchetti De Gregoris et al., 2011). Bcs-2 has been detected in the same abundance throughout cyprid development (suggesting that it may not be expressed solely as an 'early' cyprid gene as proposed by Okazaki et al., 2000); however, it was not detected in juveniles, which again implies a specific role for Bcs-2 in cyprids (Chen et al., 2014). Different expression profiles observed throughout a number of developmental stages may suggest different functional importance of the various bcs genes and their protein products; however, no specific activities or roles have been assigned (Chen et al., 2011). Therefore, it is difficult to speculate about the significance of this protein being detected in greater abundance in the colder temperature group (**Fig. 5)**, or whether it is likely to be involved in increasing adhesive strength. Though perplexing, detecting a 'cyprid specific' protein in the adult adhesive may imply greater complexity for this protein, and further investigations should be considered.

SIPC – Settlement inducing protein complex (SIPC) was the term coined to describe a larval protein complex that was implicated in the gregarious nature of barnacle settlement (Matsumura et al., 1998a; Matsumura et al., 1998b; Clare & Matsumura, 2000). These types of chemical cues were the focus of extensive research for their critical role in settlement behavior since they were

first proposed in the early 1950s (Knight-Jones & Crisp, 1953). This protein shares common sequence homology with a family of α_2-macroglobulins (family of protease inhibitors) and in fact is not a larval specific protein complex; it is found expressed in all stages of barnacle development and also in multiple tissues (Dreanno et al., 2006a).

This protein complex serves an important role in species recognition, acting as a contact pheromone that is detected by conspecific cyprid larvae as they explore potential surfaces for permanent attachment (Clare & Matsumura, 2000; Matsumura et al., 2000; Dreanno et al., 2007; Clare, 2011). This ubiquitous protein occurs in many tissues including: the cuticular tissues of both larval stages (nauplius and cypris) and adults (Dreanno et al., 2006b), the shell itself (Matsumura et al., 1998b; Dreanno et al., 2006b; Zhang et al., 2015), as well as in the temporary adhesive secretions or 'footprints' left by cyprids as they 'walk' across substrata (Dreanno et al., 2006c; Clare, 2011) suggesting its strong role in settlement, attachment, metamorphosis, and overall ecological assemblages.

Interestingly, more recent evidence suggests this protein may serve a dual role, both as a signaling molecule/settlement-inducer, as well as a putative adhesive (Petrone et al., 2015). Preliminary investigations of the adhesive properties of SIPC examined its involvement in temporary adhesion and found that although the protein shares significant sequence homology with a family of proteins that includes the α_2-macroglobulins (which are blood complement protease inhibitors, functioning to inactivate proteolytic enzymes in immune responses), it was in actuality more comparable to fibrinogen (clotting factor I), which shares no sequence homology (Petrone et al., 2015). Surface plasmon resonance (SPR) was used to measure protein adsorption behavior of SIPC on self-assembled monolayers (SAMs) compared to both α_2-macroglobulin and fibrinogen and found that SIPC behaved more similarly to fibrinogen (Petrone et al., 2015). Thus, the authors determined that SIPC appears to behave as a 'sticky' type protein, which supports its role as a proposed temporary adhesive of barnacle larvae (Petrone et al., 2015). Since SIPC is also expressed during later developmental stages, it seems probably that these sticky characteristics may also serve an additional adhesive role in more mature

barnacles. Detecting this protein in greater abundance in the colder treatment group in our study (**Fig. 5)** suggests it may contribute to stronger attachment and higher removal forces.

Serine Protease – This is a multifunctional classification of proteolytic enzymes that constitute over 50 clans and 184 families designated by the MEROPS database of known proteolytic enzymes (MEROPS release 7.90, cited by Page & Cera, 2008). With so many members, it is no surprise that the functional diversity, and also specificity, of this group of enzymes should be substantial. Emphasis placed on one family in particular (S1) describes trypsin type serine proteases involvement in a number of vital processes including blood coagulation, fibrinolysis, apopotosis, and immunity to highlight a few (Page & Cera, 2008). This is interesting with respect to a proposed model which suggested that barnacle cement polymerization is a specialized form of wound healing, biochemically similar to the enzymatic processes involved in blood clotting cascades in vertebrates and invertebrates alike (Dickinson et al., 2009).

Dickinson et al. (2009) summarized that blood coagulation in invertebrates involves similar enzymatic cascades of trypsin-like serine proteases, transglutaminase–mediated cross-linking, and proteolytic activation as found in vertebrates. Drawing on a thoroughly characterized invertebrate coagulation system involving hemolymph in horseshoe crabs (Kanost, 1999), Dickinson et al. (2009) hypothesized that barnacle hemolymph may exhibit similar serine proteinase-induced coagulation mechanisms, and further, that the coagulation of hemolymph in barnacles is linked to specialized adhesive properties. The authors concluded barnacle cement polymerization and blood clotting occur by similar mechanisms. These conclusions were evidenced through detecting the presence of biochemically similar proteins, specifically trypsin-like serine proteases and transglutaminase activity in unpolymerized adhesive (Dickinson et al., 2009).

Serine proteases have been reported in more recent studies where three different proteins containing trypsin-like serine protease domains were detected in barnacle shells using gel-based proteomics (Zhang et al., 2015). It was suggested that these particular trypsin-like

serine proteases might function to digest extracelluar matrix proteins for shell resorption, act as co-factors in the proteolytic activation of prophenoloxidase (which is implicated in wound healing and protein cross-linking), play a protective role, or perhaps be involved in the hardening process of the barnacle shell (Zhang et al., 2015). Detecting trypsin-like serine protease proteins in our study provides additional support of the coagulation cascade model of polymerization proposed by Dickinson et al. (2009), and it's increased abundance at 15°C (**Fig. 5)** also offers potential evidence for the increased adhesive strength of barnacles reared in colder temperatures.

Proteins Expressed in Higher Abundance in the 25°C Treatment Group

Neuronal Acetylcholine Receptor – Nicotinic acetylcholine receptors (AChRs) are ligand-gated cation channels that are present throughout the nervous system (Vernino, 2008). A study investigating the presence and involvement of cholinergic molecules in *B. amphirtirite* settlement and adhesion found acetylcholinesterase (AChE) activity detected in thoracic muscles, the gut wall, and cement gland of cyprids (Faimali et al., 2003). Additional detection of choline acetyltransferase-like molecules in the neuropile of the central nervous system suggested the involvement of acetylcholine in muscular contraction and cement gland exocytosis (Faimali et al., 2003). Furthermore, elevated levels of acetylcholine lead to higher settlement rates and supported the hypothesis that acetylcholine has a neurotransmitter/neuromodulator role that is important for settlement and adhesion of barnacle cyprids (Faimali et al., 2003).

Another study found AChE activity to be significantly upregulated in cyprids and also detected the presence of a neurotransmitter receptor (ie, acetylcholine receptor) with high expression levels in cyprids (Chen et al., 2014). This type of receptor binds to the neurotransmitter acetylcholine which, based on biochemical, histochemical and immunohistochemical assays, was shown to be involved in muscular contraction and cement gland exocytosis (Faimali et al., 2003). The increased expression of this receptor in cyprids provides additional evidence that acetylcholine plays a pivotal role in barnacle larval settlement (Chen et al., 2014). This protein was detected in greater abundance in the 25°C treatment group

(**Fig. 6)**, and though it has been proposed to serve an important function for settlement and possibly involved in muscular contractions of the cyprid cement gland, its role in reduced adhesion strength at warmer temperatures in adult barnacles is unclear.

Annexin – Annexins are a multigene superfamily of proteins with diverse functions (Gerke & Moss, 2002; Moss & Morgan, 2004; Rescher & Gerke, 2004; Gerke et al., 2005). In fact, this superfamily comprises more than 500 different gene products across multiple phyla and species (reviewed by Morgan & Fernandez, 1997; cited by Reschner & Gerke, 2004). Though annexins have been traditionally described as calcium-dependent phospholipid-binding proteins, more recent studies suggest increasingly complex sets of functions (Moss & Morgan, 2004). Annexins consist of a conserved core domain (thought to be the calcium regulated membrane binding module) and a secondary principal domain (NH_2-terminal which proceeds the core) that is thought to be unique between members of the family and likely responsible for individual annexin functions (Gerke & Moss, 2002). A great body of work has contributed to the general description of the biochemical and structural aspects of this protein family (Gerke & Moss, 2002). However, assigning physiological functions to the numerous members (as they are likely to differ) would provide additional insight to the potential functional diversity of the family as a whole.

The challenge resides in understanding structural differences of unique members of this large protein family, and how those differences translate to functional specificity (Moss & Morgan, 2004). Annexins have been linked to a variety of intra- and extracellular processes including (but not limited to) membrane trafficking, membrane-cytoskeleton anchorage, ion channel activity and regulation, as well as anti-inflammatory and anticoagulant activities (reviewed by Lizarbe et al., 2013). Annexin's role as an anticoagulant has been proposed for a small number of extracellular annexins (Lizarbe et al., 2013; Gerke & Moss, 2002; Gerke et al., 2005). A short description for this gene states that members of this calcium-dependent phospholipid binding family play roles as a regulator of cellular growth and signal transduction pathways, as an inhibitor of phospholipase A2, and may also play a role in anti-coagulation (summary provided by

RefSeq, Jul 2008; http://www.ncbi.nlm.nih.gov/gene/306). Intriguingly, annexins have also been detected in other barnacle studies (Chandramouli et al., 2015; Chen et al., 2014). Chen et al. (2014) showed the differential expression of one annexin protein during distinct developmental stages, where peak expression was measured nearly five times higher in the juvenile stage compared to cyprid stage.

It was hypothsized that this particular annexin might be involved in tissue degeneration and reorganization during barnacle larval development and metamorphosis (Chen et al., 2014). The annexin detected in our study shares homology with Annexin 3, which, based on early research, was identified as a type of lipocortin (inhibitor of phospholipase A2) (Tait et al., 1991), suggesting its function in anti-coagulation type activities. This “anti-coagulant” type protein perhaps provides additional support of the ‘wound healing’ model of adhesion proposed by Dickinson et al. (2009). Furthermore, this protein was found in greater abundance in the 25°C treatment (**Fig. 6**), which may explain why these animals attached with lower adhesion strengths compared to the colder reared animals in Johnston’s study (2010).

Known Cement Proteins

Though it seemed likely that a change in abundance of cement specific proteins could be responsible for the observed differences in adhesive strength reported by Johnston (2010), none of these previously published ‘cement proteins’ were detected in our analysis. However, a number of recent studies provided insight related to the temporal and mechanistic ways that adhesion occurs. Additionally, these studies discuss how some standard research methods utilized to study this complex process may lack the ability to capture some of those particular proteins.

A study investigating the process of attachment in adult barnacles demonstrated a two-step mechanism for adhesion by utilizing *in situ* optical microscopies (Burden et al., 2012). Time-lapsed microscopy showed that barnacles emit at least two optically distinct barnacle cement secretions (BCS1 and BCS2) after dislodgment and reattachment to a transparent substrate

(Burden et al., 2012). BCS2 (not to be confused with the barnacle cyprid specific gene, bcs-2, discussed earlier) is unique in that it demonstrated autofluorescent properties (characteristic of proteins) and was correlated with increased adhesion tenacity (Burden et al., 2012). Another key finding demonstrated a temporal component to BCS2 secretion that occurs over a short time frame (hours) compared to the total time required to lay down an entire new 'growth ring', which typically takes place over a number of days (Burden et al., 2012).

Additional experiments confirmed that barnacles utilize multiple means of delivering proteinaceous material to the interface (both through cuticular membranes and via capillary ducts) at different times during the growth cycle (Burden et al., 2014). Based on these findings, perhaps the appropriate window to collect a representative sample of this highly proteinaceous BCS2 secretion during our initial collection of uncured adhesive was missed. Since BCS2 was demonstrated to increase adhesive strength, conceivably this could be the main component of the uncured adhesive complex that contains a high or higher concentration of the previously described barnacle cement proteins (when compared to the first secretion, BCS1). This could explain why we did not identify these specific types of proteins during our analysis, as they may have been diluted to a level too low to be detected. Another explanation for why these proteins eluded detection could be an artifact of the collection method, and analytical techniques themselves.

Another study attempted to investigate a molt-related transcriptomic response of barnacle cement proteins in different tissues by employing a variety of collection methods and analytical techniques (Wang et al., 2015). Similar to the mass spectrometry (MS) analysis performed in our study, they too had difficulty detecting and identifying cement proteins in their MS analysis. One proposed explanation was that the cement specific proteins were masked by the presence of more abundant household and scaffold proteins and that the inability to detect the putative adhesive proteins from the uncured material does not necessarily mean that they were not present (Wang et al., 2015). In addition to lacking a standard method to collect the liquid adhesive, they suggested that the limited amount of material that can be collected during a short

time period, and the unknown possibility of cross-linking prior to enzymatic digestion for MS analysis (plus the absence of a fully annotated genome), may all contribute to the challenges for the detection of cement proteins in various barnacle secretions (Wang et al., 2015). These rationalizations illustrate the difficulties in detecting cement proteins and provide insight about the overall complexity of this unique and interesting material.

Complex Protein Matrix

A handful of proteins have been identified as functional components of the barnacle adhesive interface; however, the precise composition of these secretions relative to the entire composition of the adhering interface is still not known; thus, researchers are investigating the possibility of multiple components in the permanent adhesive processes of barnacles at different developmental stages (Burden et al., 2014). The onset of permanent attachment during the barnacle cyprid stage is in fact more complex than originally thought, involving at least two separate secretions of varying compositions of both lipids and proteins (Aldred et al., 2013; Gohad et al., 2014). Despite what is known about the functionality of a small subset of adhesive proteins, there is still much to learn, including: whether these secretions are the only source of adhesion in the interface, the exact locations of specific functional proteins, the order of their appearance and in what composition, how these compounds interact with other components in the interface, and what their overall relationship is to adhesion (Burden et al., 2014). These questions are all important aspects of the barnacle secretory mechanisms and adhesion processes that deserve further examination (Burden et al., 2014).

Summary of Findings

We detected a significant difference in protein expression between temperature groups (**Fig. 2)** and positively identified a subset of proteins (**Fig. 4)** that have the potential to play unique and unexpected roles in adhesive function. One hypothesis considered during this analysis was that perhaps the ‘cement proteins’ described in previous studies would be changing expression

due to temperature and that we could explain the differences in removal force (Johnston, 2010) by detecting a greater abundance of these very specific proteins in the adhesive of the colder temperature reared barnacles compared to the warmer temperature reared barnacles. Though we did not find these particular proteins, our study has detected and identified a number of proteins that are interesting in regards to their differential expression based on rearing temperature, and also in regards to their proposed involvement in adhesive function and tenacity.

In the 15°C treatment (higher removal force/stronger adhesive attachment), we have identified a protein that may act as a coagulation factor (trypsin-like serine protease) and another protein that may function as a putative adhesive (SIPC). Though not specific 'cement proteins,' the increased abundance of these proteins may contribute to the increased adhesion strength of barnacles attached to substrates in colder water temperatures. In the 25°C treatment (lower removal force/weaker adhesive attachment), we have identified a protein that may function as an anti-coagulant (annexin). It is feasible that the increased abundance of this protein could contribute to the weaker adhesion strength of barnacles attached to substrates in warmer water temperatures. Taken together, our findings are especially interesting in the context of the model proposed by Dickinson et al. (2009) that barnacle attachment and adhesive mechanisms are evolutionarily similar to the biochemical processes involved in 'wound healing' responses in animals. Furthermore, the differential expression of these non-cement proteins in response to temperature provide evidence that perhaps some other compounds in this complex protein matrix may also be responsible for the adhesive tenacity of this unique biofouler.

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