© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

RESEARCH ARTICLE

High spatial resolution mapping of the mucosal proteome of the gills of *Crassostrea virginica*: implication in particle processing

Emmanuelle Pales Espinosa* and Bassem Allam

ABSTRACT

In the oyster *Crassostrea virginica*, the organization of the gill allows bidirectional particle transport where a dorsal gill tract directs particles meant to be ingested while a ventral tract collects particles intended to be rejected as pseudofeces. Previous studies showed that the transport of particles in both tracts is mediated by mucus. Consequently, we hypothesized that the nature and/or the quantity of mucosal proteins present in each tract is likely to be different. Using endoscopy-aided micro-sampling of mucus from each tract followed by multidimensional protein identification technologies, and *in situ* hybridization, a high spatial resolution mapping of the oyster gill proteome was generated. Results showed the presence in gill mucus of a wide range of molecules involved in non-self recognition and interactions with microbes. Mucus composition was different between the two tracts, with mucus from the ventral tract shown to be rich in mucin-like proteins, providing an explanation of its high viscosity, while mucus from the dorsal tract was found to be enriched in mannose-binding proteins, known to be involved in food particle binding and selection. Overall, this study generated high-resolution proteomes for *C. virginica* gill mucus and demonstrated that the contrasting functions of the two pathways present on oyster gills are associated with significant differences in their protein makeup.

KEY WORDS: Bivalve, Mucus, Proteomic, Mass spectrometry, Feeding, Particle selection

INTRODUCTION

Living organisms are unceasingly exposed to challenging physical, chemical and biological factors, so that their fitness, and often their survivorship, relies on the efficiency of the barriers they have built. In the case of the metazoa, mucosal secretions associated with epithelial layers represent the first line of defense against various attacks. Mucus is secreted by all epithelia containing living cells on their surfaces such as on the internal organs of vertebrates (e.g. gastrointestinal or tracheobronchial tracts) and the epidermis of fish, amphibians, cnidarians and mollusks. It is made of mucin-like molecules, water, electrolytes, epithelial and blood cells and a wide range of bioactive molecules produced by mucus-secreting cells (Schachter and Williams, 1982; Simkiss and Wilbur, 1977). The consistency, viscosity and elasticity of mucus are generally attributed to the concentration of polymers (e.g. mucopolysaccharides, mucins, mucin-like glycoproteins) within the gel and to the physical entanglement of these polymers with other molecules (Cone, 2009; Rose et al., 1984; Smith and Morin, 2002).

In mollusks, mucus has a central role in multiple biological functions (Davies and Hawkins, 1998), including locomotion and navigation (Denny, 1989; Prezant and Chalermwat, 1984; Smith et al., 1999), attachment (Smith, 2002), protection against freezing (Hargens and Shabica, 1973) or desiccation (Denny, 1989; Wolcott, 1973), and defense against predators (Gavagnin et al., 1994; Gustafson and Andersen, 1985). The energy allocated to mucus production in mollusks can exceed 15% of the total energy gained from food, highlighting the importance of mucus in the biology of these animals (Davies and Hawkins, 1998). One of the most important biological functions of mucus in bivalve mollusks (e.g. oysters, mussels, clams) is interaction with microbes (reviewed by Allam and Pales Espinosa, 2015). For instance, the mucus layer covering the surface of bivalve pallial organs (organs present in the shell cavity such as gills, mantle) is the first constituent encountered by waterborne microbes that attach to these organs before the establishment of mutualistic (symbionts) or parasitic associations (Allam et al., 2013; Burreson and Ford, 2004; Dahl et al., 2010; Dubilier et al., 2008). Furthermore, previous studies have shown that molecules present in mucus contribute to the establishment and the success or failure of many of these host–microbe associations (Allam et al., 2013; Dufour, 2005; Kremer et al., 2013; Pales Espinosa et al., 2014, 2013; Southward, 1986).

Mucus is also commonly used by mollusks to capture and transport particles on ciliated epithelia for cleansing and feeding (Barille and Cognie, 2000; Beninger et al., 1993; Morton, 1977; Urrutia et al., 2001). In suspension-feeding bivalves, particles are captured by the gills, embedded in mucus and transported on the feeding organs (i.e. gills, labial palps) to be either rejected as pseudofeces or directed to the mouth and ingested (Beninger et al., 1993; Urrutia et al., 2001; Ward et al., 1993). This food particle sorting mechanism has been well described for over a century and is considered to represent an important strategy allowing bivalves to optimize energy gain by ingesting nutrient-rich particles while rejecting poor quality ones in pseudofeces (Allen, 1921; Bayne et al., 1993; Cognie et al., 2001; Newell and Jordan, 1983). Although the precise mechanism of sorting in suspension-feeding bivalves remains unclear, *in situ* observations demonstrated that mucus covering bivalve feeding organs plays an important role in particle processing as a vehicle for particle capture, post-capture transport, ingestion and rejection steps (Beninger et al., 1993; Riisgard et al., 1996). Particles directed as pseudofeces are embedded in cohesive mucus and rejected back into the environment via a ventral tract entangling unwanted live cells, debris, and abiotic material of low nutritional value. Those directed for ingestion are transported to the mouth via a dorsal tract in a low viscosity mucus (Beninger et al., 1992; Ribelin and Collier, 1977). But mucus is not just a mere carrier for food particles: recent investigations showed that specific interactions take place between mucus and food particles, mediating particle selection. In this context, our previous work

School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794-5000, USA.

*Author for correspondence (emmanuelle.palesespinosa@stonybrook.edu)

E.P.E., 0000-0003-1779-6757; B.A., 0000-0003-1278-7915

Received 15 July 2020; Accepted 6 January 2021

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

demonstrated that mucus covering the feeding organs of the eastern oyster *Crassostrea virginica* (Pales Espinosa et al., 2009) contains sugar-binding proteins (i.e. lectins) that differentially bind microalga cell surface carbohydrates (MCSCs), triggering particle selection (Pales Espinosa and Allam, 2018). Moreover, a series of feeding experiments showed that oysters preferentially ingest particles covered with glucose and mannose residues, as a likely result of interactions between MCSCs and mucosal lectins present on their feeding organs (Pales Espinosa et al., 2016a).

A low-resolution reference map of proteins found in pallial mucus covering the gills, labial palps and mantle of *C. virginica* has already been generated, highlighting the presence of a wide variety of putative adhesion/recognition molecules (Pales Espinosa et al., 2016b). Although this first analysis reveals the presence of multiple lectins, the sampling approach used generated bulk mucus from each organ without the spatial resolution needed to gather information about the specific role of mucus in particle selection. The current study was designed to generate a high spatial resolution of the proteomic composition of the mucus that covers the gills of *C. virginica*, with an emphasis on the ventral (i.e. associated with the rejection of particles) and dorsal tract (i.e. associated with the ingestion of particles). This was mainly accomplished using endoscopy-aided micro-sampling of mucus from each tract *in vivo*, followed by multidimensional protein identification technology and complemented by *in situ* hybridization of candidate proteins. Our working hypothesis was that the functional disparities between both gill tracts is the result of differences in the proteomic make-up of mucus present in each tract.

MATERIALS AND METHODS

Mucus collection

Adult *Crassostrea virginica* (Gmelin 1791) (80–100 mm in length, n=15) were obtained from a commercial source located on Long Island Sound, NY, USA in September 2014, cleaned of epibionts and maintained in a flow-through system using natural seawater pumped from Long Island Sound (~20°C) until use. To prepare animals for the procedure, a small section of the inhalant margin of the upper and lower valves of each specimen was carefully trimmed without damaging the underlying mantle tissue. Oysters were then allowed to recover for at least 1 day before mucus collection. During the mucus collection procedure, bivalves were placed in an aerated assay chamber (~1 liter) filled with filtered seawater at ambient temperature (~20°C). Mucus samples were collected *in vivo* using a micropipette connected to a peristaltic pump. The sampling pipette was mounted on a micromanipulator and positioned with the aid of an endoscope (for complete procedure, see Ward et al., 1991, 1998). Mucus samples were collected from the ventral and dorsal tract of the gills (Fig. 1B,F) and kept on ice during the procedure. Simultaneously, a cocktail of general protease inhibitors (50 µl of 1× solution prepared following manufacturer's recommendation per 50 ml mucus, S8820, Sigma) was added into each sample during the collection to prevent protein degradation. A total of 22 samples (i.e. 11 from each tract, volume ranging from 5 to 57.5 ml, Table S1) were collected, immediately frozen and stored at −80°C until analysis that happened within a week.

Proteomic sample preparation

Before protein analysis, samples from the same tract were randomly combined (final volume ranging from 80 to 97.5 ml) into a total of 3 pools (Table S1) and concentrated by filtration (Amicon Ultra-15 Centrifugal Filter Units with Ultracel-3 membrane, Millipore, Burlington, MA) as per the manufacturer's recommendations. A quality control step was implemented to check protein abundance. An aliquot (25 µl) was mixed with 25 µl of 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) heated to 100°C for 10 min and separated on a precast 12% Tris-Glycine gel (Jule Biotechnologies, Inc., Milford, CT). After electrophoresis, gels were stained using a standard silver stain protocol. The volume of the concentrated protein solution was finally reduced to 100 µl using a Speed-Vac. Non-protein components were removed from the protein solution by deoxycholate-TCA precipitation using a modification of the method of Peterson (1977). The resultant protein pellet was dissolved in 20 µl 8 mol l^{-1} urea, 25 mmol l^{-1} NH_4HCO_3. Protein concentrations were determined using the Peterson modification of the Lowry assay (Peterson, 1977). The protein solution was subjected to trypsin digestion as follows: reduced in 4 mmol l^{-1} DTT (30 min, room temperature), alkylated in 8.4 mmol l^{-1} iodoacetamide (30 min, room temperature in the dark), before the urea concentration was reduced to 1.7 mol l^{-1} and the solution incubated 16 h at 37°C in the presence of trypsin Gold (Mass Spectrometry Grade, Promega, Madison, WI) at >1 µg/40 µg protein. After incubation, the digest was added with 2% formic acid.

Mass spectrometry and data analysis

The samples were analyzed for protein content using a modification of the multidimensional protein identification technology (MUDPIT) method (Washburn et al., 2001). Samples were pressure bomb loaded through the proximal end of a 'mudpit' column constructed of 250 µm ID fused silica tubing (PT Polymicro Technologies, Phoenix, AZ) with Kasil frit at distal end. The column was packed with 3 cm of strong cation exchanger (SCX, 5 µm) matrix (Whatman) distally and 3 cm C_{18} matrix (5 µm ProntoSil 120-5-C18H, Bischoff Chromatography, Leonberg, Germany) proximally. Following sample loading, the column was washed for 10 min with Buffer A [2% acetonitrile (ACN), 0.1% formic acid (FA)] at 300 nl min^{-1}. The mudpit column was connected with a microtee to a fritless electrospray interface (Gatlin et al., 1998) feed column for automated microcapillary liquid chromatography-tandem mass spectrometry. The nano electrospray feed column to the mass spectrometer consisted of a fused-silica capillary (100 µm ID) which was pulled using a P-2000 CO_2 laser puller (Sutter Instruments, Novato, CA) to a 5 µm ID tip and packed with 10 cm of 5 µm ProntoSil C18 matrix using a pressure bomb and subsequently equilibrated in Buffer A.

The dual column construct was placed in line with an Eksigent 2D NanoHPLC unit flowing at 300 nl min^{-1}. The HPLC separation was provided by a 13 step, three component gradient. Each step consisted of the following, in sequence: 5 min wash with 100% Buffer A; 5 min wash with a fixed percentage of Buffer C (0.5 mol l^{-1} ammonium acetate, in Buffer A); 10 min wash with 100% Buffer A; 60 min gradient of 0% to 40% Buffer B (90% ACN, 0.1% FA); 30 min wash, 100% Buffer A. The 13 steps varied the fixed Buffer C from 0 to 100%. The application of a 2.2 kV distal voltage electrosprayed the eluting peptides directly into an LTQ Orbitrap XL ion trap mass spectrometer (Thermo Fisher, San Jose, CA). Full mass spectra (MS) were recorded on the peptides over a 400 to 2000 m/z range at 60,000 resolution, followed by five tandem mass (MS/MS) events sequentially generated in a data-dependent manner on the first, second, third, fourth and fifth most intense ions selected from the full MS spectrum (at 35% collision energy). Charge state dependent screening was turned on, and peptides with a charge state of +2 or higher were analyzed. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Fisher, San Jose, CA).

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

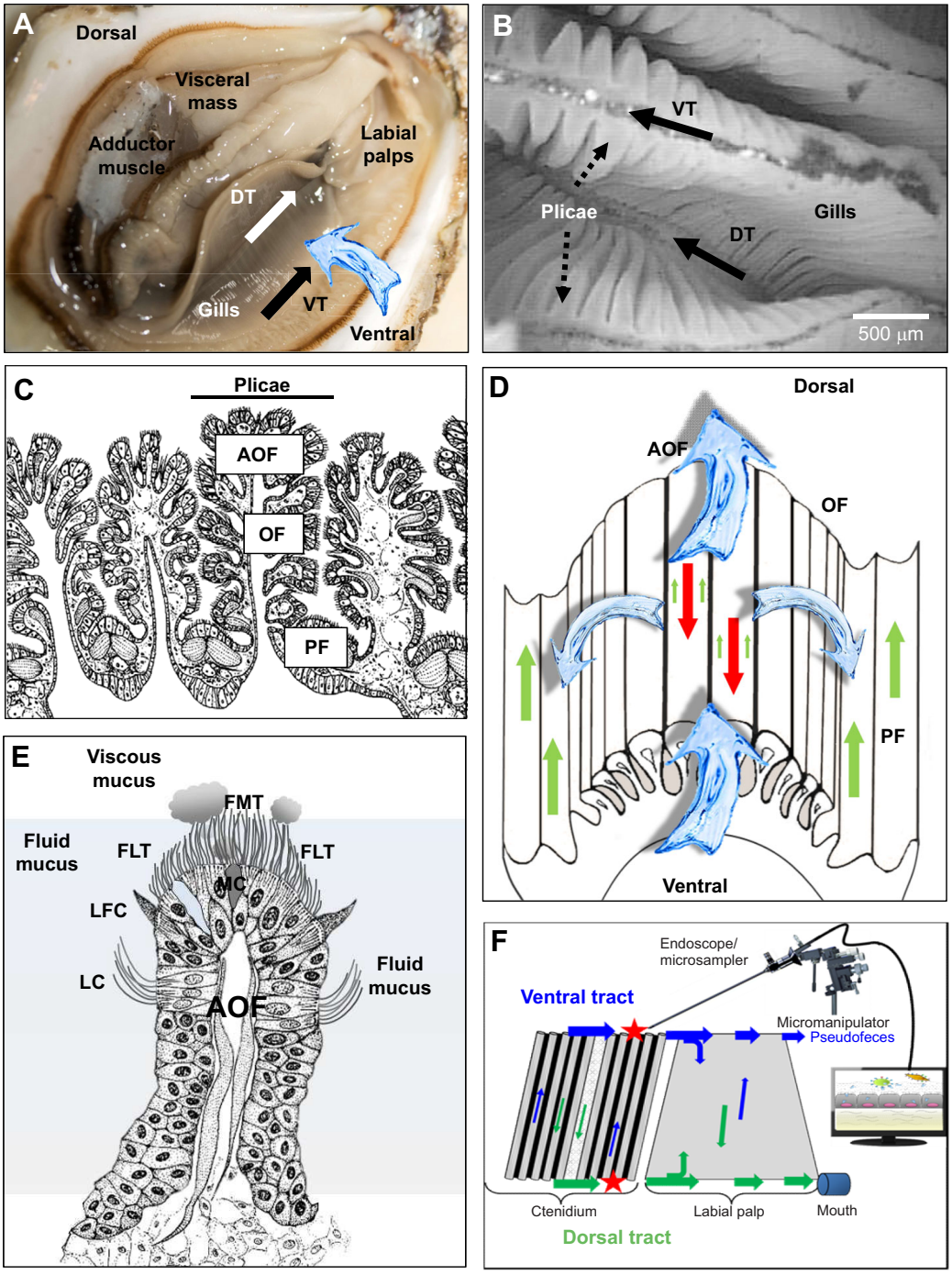

Fig. 1. Images and schematic drawings of the oyster *Crassostrea virginica* and its mucus collection apparatus. (A) The principal organs of *C. virginica* including the gills, the labial palps, the adductor muscle and the visceral mass. Blue arrow indicates the direction of the water flow entering the pallial cavity. (B) Magnified view of the ventral tract (VT) and the dorsal tract (DT). (C–F) Each gill plica comprises principal filaments (PFs) and several ordinary filaments (OFs), including apical ordinary filaments located at the apex of the plicae (AOFs, detailed in E). PFs harbor cilia beating dorsally (transport symbolized by the green arrows in D) while cilia present on AOFs perform bi-directional transport (red arrows indicate cilia beating ventrally while the green ones indicate cilia beating dorsally). Blue arrows indicate direction of water flow. The different types of cilia present on the AOFs (E) include the lateral cilia (LC), the latero-frontal cilia (LFC), fine cilia forming the frontal lateral tract (FLT) and coarse cilia forming the frontal median tract (FMT). Viscous mucus (gray aggregates in E) is secreted by mucocytes (MC, shown in dark gray) below the FMT while fluid mucus (blue area) is secreted by mycocytes (MC, shown in light gray) below the FLT. The red stars in F represent the sites of mucus collection from each tract. The image in B is courtesy of B. Cognie; C and E are redrawn from Galtsoff, 1964 and Beninger et al., 2005.

MS/MS spectra were extracted from the RAW file with ReAdW.exe (http://sourceforge.net/projects/sashimi). The resulting mzXML data files were searched with The GPM X! Tandem (The GPM, thegpm.org; version CYCLONE 2013.02.01.1) against a custom proteome database (48,093 entries) built using the *Crassostrea virginica* open reading frames produced from the oyster transcriptome generated by McDowell

Journal of Experimental Biology

et al. (2014). This approach excludes proteins potentially derived from microbes associated with pallial mucus. Fixed cysteine carbamidomethylation and optional methionine oxidation and threonine, serine and tyrosine phosphorylation were applied during the search. Only peptides with a P value of ≤0.01 were analyzed further. In addition, a Decoy database (all proteins in reverse order) was also added from this database with compass (Wenger et al., 2011). This database was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da. Scaffold (v.4.4.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 5.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. The sequences of the proteins identified in the 2 mucosal tracts were then uploaded into the Blast2GO application (Götz et al., 2008) to be annotated.

RNAscope *in situ* hybridization

Adult *C. virginica* (80–100 mm in length, n=6) were obtained from a commercial source located on Long Island Sound, NY, in November 2017. Oyster gills were dissected and fixed in 10% neutral buffered formalin for 48 h before being dehydrated in an ascending ethanol series, embedded in paraffin blocks and cut in serial sections (5 µm thickness). Four consecutive sections were processed for standard Hematoxylin and Eosin staining (1 section) or for *in situ* hybridization (ISH, 3 sections). RNA *in situ* hybridization assays were performed using RNAscope®, an RNA *in situ* hybridization technique previously described by Wang et al. (2012). The RNAscope® 2.5 HD Red Reagent Kit (Advanced Cell Diagnostics, Newark, CA) was applied in accordance with manufacturer's instructions. Three probes custom-synthesized by the manufacturer were used: one against the mucosal C-type lectin (CvML3912, Pales Espinosa and Allam, 2018), chosen because of its abundance in oyster mucus and its demonstrated role in particle selection in oyster; a control probe targeting oyster 18S (Cv18S-rRNA; X60315.1) was used to assess RNA integrity and evaluate ISH reaction success; a negative control probe specific to the bacterial dapB gene (EF191515) was also used to evaluate non-specific binding and background staining. Slides were then counterstained and observed using a microscope. Thirteen plicae were selected to enumerate CvML3912-positive cells on the apex [4 apical ordinary filaments (AOFs)] and on the side [principal filament (PF) and lateral ordinary filaments (LOFs)] of each plica (Fig. 1C).

Statistical analysis

Statistical analysis compared protein composition in mucus collected from the ventral and dorsal tracts. For downstream analysis, only proteins for which at least 2 unique peptides were identified, that were present in two out of six samples, and that presented a $\log_e$ value <−9 were considered (40% of the initial proteins). Standardization to the sum of proteins identified in the corresponding sample was realized and protein abundance data were analyzed in MultiExperiment Viewer software (MeV, v.4.9). Significance Analysis of Microarray (SAM) methods were used to identify proteins differentially abundant in samples from the different tracts following the approach described by Roxas and Li (2008). A gene ontology (GO) enrichment analysis was performed in Blast2GO using the Fisher's Exact Test (P value of 0.05 as cut-off) to compare protein found in the ventral and dorsal tracts against the total proteome.

RESULTS AND DISCUSSION

Several proteomics studies on mollusk shell (Mann and Jackson, 2014; Marie et al., 2012) and mucosal secretions (Caruana et al., 2016;

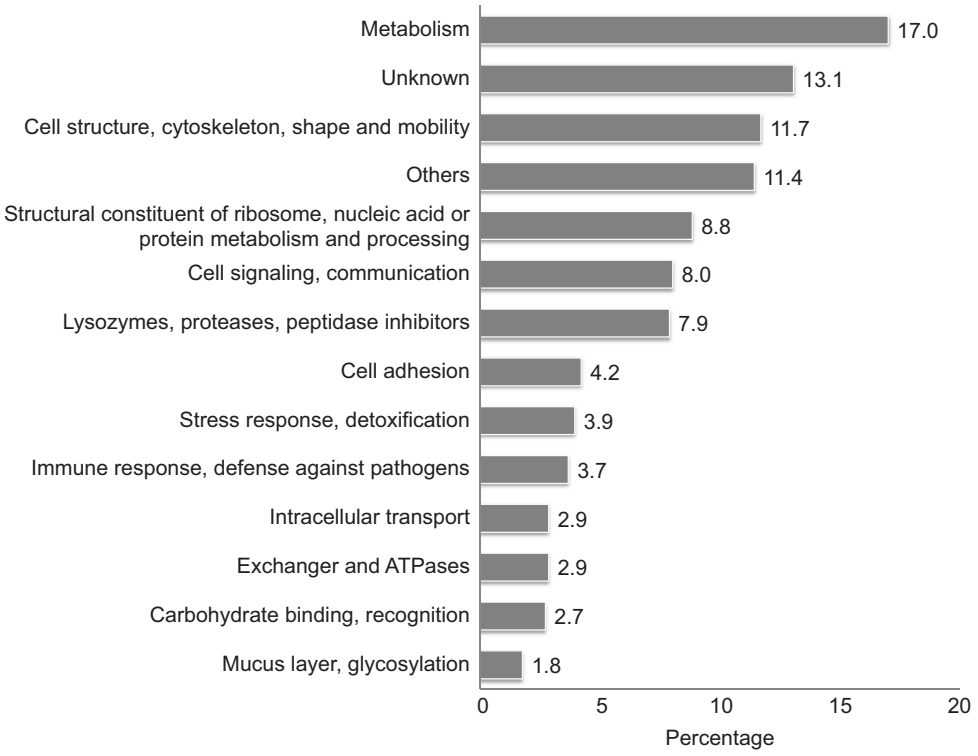

Fig. 2. Functional classification of the proteins (represented as percentage of the total) identified in the pallial mucus of *C. virginica*.

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

Table 1. Twenty most abundant proteins in the ventral and dorsal tracts of the gills of the oyster *Crassostrea virginica*

Accession no.	Sequence description	Best hit	Ventral	Dorsal
cds.c107101_g1_i6|m.9004	Extracellular superoxide dismutase	BAF30874	16,995	6077
cds.c107101_g1_i1|m.8995	Extracellular superoxide dismutase	BAF30874	–	10,922
cds.c107101_g1_i2|m.8997	Extracellular superoxide dismutase	BAF30874	1770	1628
cds.c107101_g1_i5|m.9003	Extracellular superoxide dismutase	BAF30874	1411	1009
cds.c116626_g1_i1|m.32008	Major plasma protein 2	AFH41574	1313	1565
cds.c107101_g1_i4|m.9001	Extracellular superoxide dismutase	BAF30874	554	374
cds.c104885_g1_i1|m.6117	Carbonic anhydrase 2-like	XP_011434938	477	955
cds.c116626_g3_i1|m.32012	Major plasma protein 2	AFH41574	257	238
cds.c114486_g3_i1|m.25526	Actin	AEF33434	212	175
cds.c119321_g2_i1|m.41766	Tubulin alpha-1a chain-like isoform 2	XP_002738175	179	97
cds.c125643_g1_i2|m.71275	Sco-spondin precursor	EKC38294	172	123
cds.c114868_g1_i1|m.26640	X-box binding protein	AEF33390	137	–
cds.c105530_g1_i1|m.6886	SCO-spondin	EKC38295	134	–
cds.c111275_g1_i4|m.16955	Hypothetical protein CGI_10026433	EKC41815	121	104
cds.c125842_g3_i1|m.72774	Tubulin beta chain isoform x1	XP_004226974	112	80
cds.c106651_g1_i1|m.8346	Hypothetical protein CGI_10026432	EKC41814	106	–
cds.c105863_g2_i2|m.7331	Hypothetical protein CGI_10007012	EKC32514	–	99
cds.c119691_g2_i1|m.43169	Hypothetical protein CGI_10023805	EKC29415	95	90
cds.c112731_g2_i1|m.20635	Hypothetical protein CGI_10004310	EKC27276	93	–
cds.c102634_g1_i3|m.3915	C-type mannose receptor 2	EKC30902	–	92
cds.c112703_g1_i1|m.20523	Calmodulin-like isoform 4	EGI70237	91	–
cds.c122613_g1_i1|m.55693	Aminopeptidase-like	EKC23074	88	–
cds.c102634_g1_i2|m.3914	C-type mannose receptor 2	EKC30902	–	83
cds.c98517_g1_i1|m.1845	60 s ribosomal protein l40a	AFI80900	–	79
cds.c101519_g1_i1|m.3174	Lysozyme 2	Q1XG90	–	78
cds.c113708_g1_i1|m.23265	L-ascorbate oxidase	EKC33283	70	152

Minus symbol (–) indicates that the protein is not among the top 20 expressed in each tract.

Pales Espinosa et al., 2016b) have provided reference maps allowing for further exploration of suspected processes and functions attributed to mucosal proteins. In this new study, a total of 1833 proteins matching *C. virginica* predicted proteins were identified in samples collected from the two mucosal tracts combined (Table S2). Several stringent selection criteria (see Materials and Methods) yielded 735 selected proteins that were finally grouped into 14 categories (Fig. 2) based on their functional annotation (NCBI database) and a complementary search using Blast2go (GO terms, Enzyme Codes, IPR). Some of these proteins have an intracellular origin (e.g. tubulin or ribosomal proteins) likely because of the presence in the pallial mucus of hemocytes and exfoliated epithelial cells, but also to the transudation of plasma components into pallial mucus. In contrast, 56 of these 735 proteins match the GO terms 'extracellular', 'cell-matrix adhesion' and 'integral to membrane' and 155 additional ones present a signal peptide or a transmembrane domain (SignalP-TM; Table S3) suggesting that these particular proteins are secreted. Among the most abundant proteins present in the combined tracts of *C. virginica* gills (Table 1), the dominin (extracellular superoxidase dismutase, BAF30874; Itoh et al., 2011; Xue et al., 2019) and its isoforms represent about 60% of the total spectral counts. The major plasma protein 2 (also known as segon, AFH41574; Xue et al., 2019, 2012) is the second most abundant protein with 5.6% of the total spectral counts. This finding is in agreement with a previous study showing that the two proteins represent about 70% of the total proteins from oyster hemolymph (Xue et al., 2019). They are both suspected to be involved in shell formation, possibly explaining their presence in pallial mucus (Xue et al., 2019). The 20 most abundant proteins in both tracts also include a carbonic anhydrase 2-like (2.3% of the total spectral count, XP_011434938) as well as proteins involved in cytoskeletal filaments structure (e.g. actin, tubulin, calmodulin-like), cell matrix formation (e.g. SCO-spondin), adhesion or recognition (e.g. x-box binding, c-type mannose receptors 2), proteolysis or cytolysis (e.g. aminopeptidase-like, lysozyme 2), as well as several hypothetical proteins.

While most of the 735 proteins identified in mucus were common to both tracts, the relative abundance of 56 proteins was found to be significantly different between both sample types (i.e. the ventral tract versus dorsal tract, Table 2, Table S4). Among the 56 tract-specific proteins, the abundance of 34 was significantly higher in the dorsal tract compared to the ventral, while 22 proteins were higher in the latter (Table 2). An enrichment analysis showed that two categories (i.e. 'carbohydrate binding, recognition' and 'mucus layer') were particularly enriched compared to others (Fig. 3). These proteins were grouped based on available information and are discussed from the lens of oyster interaction with waterborne microbes (e.g. particle transport and selection process, microbial neutralization and digestion).

Particle transport and selection process

In the present study, results show that the mucus in the ventral tract is characterized by a high abundance of 12 SCO-spondin/mucin-like, with 3 of these proteins being significantly higher than levels detected in the dorsal tract (GO terms 'mucus layer, glycosylation', Table 2, Table S3). The overall standardized spectral count of mucin-like proteins reached 483 in the ventral tract versus 273 in the dorsal tract. In addition to the mucin-like proteins, mucus from the ventral tract is also characterized by the presence of a large number of proteins with adhesive property. For instance, the normalized total count of proteins with the GO term 'cell adhesion' was 63% higher in the ventral tract (465 spectral counts, Table S3) than in the dorsal (296 spectral counts), with the presence of numerous proteins containing von Willebrand factor (VWA) and EGF domains, including matrilin-like protein as well as a significant enrichment in galactose-binding lectin (galactoside-specific lectin) and fucose-binding lectin (fucolectin-7-like; Table 2, Table S3).

In contrast, the mucus from the dorsal tract is particularly enriched in proteins characterized by the GO term 'carbohydrate binding'

Table 2. Average spectral counts (±s.e.) of the 56 differentially abundant proteins in mucus derived from the ventral and dorsal tracts of the gill

Accession no.	Sequence description	Best Hit	*E*-value	Gene ontology (GO) annotation	Average spectral counts		Adj. *P*-value
					Ventral	Dorsal	
Mucus layer, glycosylation							
cds.c105530_g1_i1|m.6886	SCO-spondin	EKC38295	5.04E-21	–	**44.7±13.2**	17.4±1.0	0.048
cds.c108241_g1_i1|m.10701	SCO-spondin	EKC38295	4.68E-29	–	**16.0±3.0**	4.0±0.5	0.003
cds.c51802_g1_i1|m.134	SCO-spondin	EKC38295	2.14E-27	–	**2.3±1.0**	0.2±0.2	0.045
Carbohydrate binding, recognition							
cds.c124078_g2_i1|m.62523	C-type lectin 2 like protein	AJ579379	2.00E-36	F:carbohydrate binding	1.8±0.4	**10.0±3.0**	0.018
cds.c102634_g1_i2|m.3914	C-type mannose receptor 2	EKC30902	2.23E-58	F:carbohydrate binding	6.9±1.2	**27.7±9.1**	0.020
cds.c102634_g1_i3|m.3915	C-type mannose receptor 2	EKC30902	3.49E-53	F:carbohydrate binding	2.4±2.4	**30.8±9.1**	0.048
cds.c107314_g1_i2|m.9345	D-galactoside-specific lectin	EKC22364	1.84E-35	–	**6.3±3.4**	0.0±0.0	0.002
cds.c102451_g3_i1|m.3776	Fucolectin-7-like	XP_011426188	6.00E-57	F:carbohydrate binding	**5.8±1.5**	0.7±0.7	0.049
Cell adhesion							
cds.c119148_g1_i1|m.41188	IgGF-C-binding protein	EKC36905	2.71E-208	P:cell-matrix adhesion	0.2±0.2	**2.4±0.4**	0.027
cds.c123002_g1_i1|m.57592	Cell adhesion molecule 4	EKC31047	3.47E-116	(Ig superfamily)	0.0±0.0	**3.0±1.6**	0.007
cds.c115021_g1_i1|m.27136	von Willebrand factor type EGF and pentraxin domain-containing protein	EKC35876	4.15E-182	–	**8.3±1.0**	0.2±0.2	0.005
Lysozymes, proteases, peptidase inhibitors							
cds.c125270_g1_i1|m.69063	Kyphoscoliosis peptidase	EKC37229	3.90E-170	–	0.0±0.0	**2.5±0.4**	<0.001
cds.c122769_g1_i1|m.56421	Tolloid-like protein 2	EKC26355	0	P:proteolysis; F:serine-type endopeptidase activity; F:catalytic activity; F:serine-type peptidase activity; F:hydrolase activity; F:peptidase activity	0.0±0.0	**3.0±1.7**	0.009
cds.c125370_g1_i1|m.69610	Kazal-type serine proteinase inhibitor	ACD88987	2.06E-53	P:proteolysis; F:peptidase activity	**6.5±0.7**	0.7±0.4	0.037
cds.c104568_g1_i1|m.5769	Metalloproteinase inhibitor 1-like	EKC31955	6.11E-16	F:metalloendopeptidase inhibitor activity; C:extracellular region	**8.4±3.7**	0.2±0.2	0.010
cds.c102468_g1_i1|m.3788	Pancreatic trypsin inhibitor	XP_001360711	4.42E-05	F:serine-type endopeptidase inhibitor activity	**0.8±0.1**	0.0±0.0	<0.001
Structural constituent of ribosome, nucleic acid and protein metabolism and processing							
cds.c113962_g1_i1|m.23988	Low quality protein: 7sk snRNA methylphosphate capping enzyme	ELU07128	5.43E-66	F:methyltransferase activity; P:methylation	0.0±0.0	**1.1±0.3**	<0.001
cds.c113354_g1_i1|m.22291	Poly ADP-ribose polymerase family member 14	XP_003795161	4.07E-18	F:NAD^+ ADP-ribosyltransferase activity	0.0±0.0	**0.7±0.1**	<0.001
cds.c122603_g1_i1|m.55648	Transcription factor ap-2 epsilon	EKC26960	0	–	0.5±0.5	**3.7±0.7**	0.045
cds.c115774_g1_i1|m.29324	tRNA (uracil-5-)-methyltransferase-like protein a	EKC32655	1.60E-224	F:heterocyclic compound binding; F:organic cyclic compound binding	0.8±0.1	**2.0±0.5**	0.040
Cell structure, cytoskeleton, shape and mobility							
cds.c113381_g1_i1|m.22332	Collagen alpha-6 chain	EKC21647	1.16E-74	–	1.8±0.6	**16.1±5.2**	0.007
cds.c114415_g2_i1|m.25328	Collagen alpha-6 chain	EKC33510	5.38E-33	–	0.0±0.0	**1.9±0.6**	0.001
cds.c118038_g1_i1|m.36967	S-crystallin sl11	EKC43008	1.20E-28	F:actin binding	2.2±0.7	**6.5±1.1**	0.046
cds.c116822_g1_i2|m.32613	Tubulin polymerization-promoting protein family member 3	EKC17872	8.29E-53	–	4.2±2.0	0.0±0.0	0.007
Immune response, defense against pathogens							
cds.c110839_g1_i1|m.15938	Complement c1q tumor necrosis factor-related protein 3	EKC38694	2.10E-64	–	0.5±0.5	**8.0±4.7**	0.039
cds.c122006_g1_i2|m.52775	Beta-glucan-binding protein 1	EKC31577	3.61E-83	F:hydrolase activity, hydrolyzing O-glycosyl compounds; P:carbohydrate metabolic process	0.2±0.2	**7.5±3.6**	0.017
Cell signaling, communication							
cds.c125904_g1_i1|m.73302	Polycystin-1-like isoform x1	EKC35176	0	P:neuropeptide signaling pathway; F:calcium ion binding; C:integral to membrane; C:membrane	0.0±0.0	**2.2±1.0**	0.005
cds.c100478_g1_i1|m.2579	Tbc1 domain family member 13	EKC42656	1.22E-56	–	0.0±0.0	**3.7±2.4**	0.009

Continued

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

Table 2. Continued

Accession no.	Sequence description	Best Hit	*E*-value	Gene ontology (GO) annotation	Average spectral counts: Ventral	Average spectral counts: Dorsal	Adj. *P*-value
cds.c125941_g1_i1|m.73591	Receptor-type tyrosine-protein phosphatase t	EKC33205	2.68E-206	–	**13.3±5.1**	1.9±0.9	0.026
Stress response, detoxification							
cds.c108750_g3_i1|m.11638	Thioredoxin peroxidase	ADC35419	1.40E-95	–	**3.0±1.0**	0.0±0.0	0.002
Exchanger and ATPases							
cds.c113552_g2_i1|m.22835	NADPH-cytochrome p450 reductase	EKC32653	7.69E-48	–	0.0±0.0	**2.6±1.1**	0.001
cds.c114608_g3_i1|m.25874	ATP synthase subunit mitochondrial	EKC39411	2.97E-58	F:proton-transporting ATP synthase activity, rotational mechanism; P:ATP hydrolysis coupled proton transport; C: proton-transporting ATP synthase complex, catalytic core F(1); P:ATP synthesis coupled proton transport; F:ATP binding	**2.0±0.8**	0.0±0.0	0.001
Intracellular transport							
cds.c105598_g1_i1|m.7000	Endoplasmic reticulum-resident calcium binding protein	XP_005109029	5.90E-06	F:calcium ion binding; C:endoplasmic reticulum; P:intracellular protein transport	2.5±1.3	**16.0±3.8**	0.021
cds.c123869_g1_i1|m.61515	GDP-fucose transporter 1	EKC31466	1.81E-177	C:integral to membrane	0.0±0.0	**2.9±1.1**	0.005
cds.c97897_g1_i1|m.1665	Sodium-coupled neutral amino acid transporter partial	EKC35772	2.32E-43	–	**3.2±0.4**	0.0±0.0	<0.001
cds.c116696_g1_i1|m.32238	Sodium-dependent phosphate transport protein 2b	EKC36781	5.22E-194	–	**12.5±4.4**	0.7±0.5	0.025
Metabolism							
cds.c122193_g2_i2|m.53708	Adam family mig-17	EKC23719	5.76E-107	F: metalloprotease activity	0.0±0.0	**3.9±0.8**	<0.001
cds.c122884_g1_i1|m.56982	Beta-galactosidase-1-like protein 2	EKC24362	7.06E-199	F:hydrolase activity	1.2±1.2	**14.7±6.4**	0.049
cds.c104885_g1_i1|m.6117	Carbonic anhydrase 2-like	XP_011434938	4.00E-150	–	158.9±6.8	**318.4±53.3**	0.022
cds.c112408_g1_i2|m.19831	Chitinase 3	EKC38803	4.50E-121	–	0.0±0.0	**0.7±0.1**	<0.001
cds.c122741_g1_i1|m.56294	Carboxylesterase 1C-like	XP_011423382	0	–	0.0±0.0	**1.3±0.3**	0.005
cds.c115143_g1_i2|m.27502	Di-N-acetylchitobiase	EKC21732	1.69E-186	–	0.2±0.2	**2.9±1.0**	<0.001
cds.c117341_g1_i2|m.34272	Glucose dehydrogenase	EKC40457	1.64E-31	–	0.0±0.0	**2.2±1.2**	0.033
cds.c110199_g1_i1|m.14549	Peritrophin-1-like	EKC31969	2.82E-51	F:chitin binding; P:chitin metabolic process; C:extracellular region	**3.7±2.0**	0.0±0.0	0.002
cds.c125632_g1_i1|m.71211	Uncharacterized protein loc101848577	EKC41432	0	F:chitin binding; P:chitin metabolic process; F:calcium ion binding; C:extracellular region	**16.4±1.2**	4.5±0.6	0.001
Others							
cds.c125721_g1_i1|m.71819	Kinesin-like protein partial	EKC20730	0	–	0.0±0.0	**0.9±0.3**	0.001
cds.c96074_g1_i1|m.1296	Sarcoplasmic calcium-binding protein	EKC29121	5.69E-86	F:calcium ion binding	2.1±0.8	**11.2±2.9**	0.032
cds.c117274_g1_i1|m.34011	Calmodulin mutant syncam9	EKC42677	2.16E-57	F:calcium ion binding	**4.0±1.1**	0.4±0.4	0.038
cds.c103916_g1_i1|m.5012	Protein dj-1	EKC37254	2.10E-82	–	**1.3±0.1**	0.2±0.2	0.041
cds.c105863_g2_i2|m.7331	Hypothetical protein CGI_10007012	EKC32514	1.78E-45	(uncharacterized)	3.5±1.0	**32.9±19.0**	0.039
cds.c116398_g1_i1|m.31244	Hypothetical protein CGI_10018743	EKC30064	5.75E-54	–	0.0±0.0	**3.6±1.4**	0.001
cds.c107863_g1_i1|m.10153	Hypothetical protein CGI_10027195	EKC36298	4.75E-64	(EF-hand domain)	0.2±0.2	**12.0±2.7**	0.006
cds.c122231_g3_i1|m.53912	–			–	0.0±0.0	**4.3±2.1**	0.002
cds.c109620_g1_i1|m.13261	Hypothetical protein CGI_10017882	EKC26650	1.55E-115	–	**5.1±1.3**	0.0±0.0	<0.001
cds.c113868_g1_i2|m.23685	Hypothetical protein CGI_10019816	EKC30399	1.29E-81	(uncharacterized)	**12.8±3.9**	3.2±1.1	0.035
cds.c109341_g1_i1|m.12686	–			–	**2.7±1.5**	0.0±0.0	0.004

Bold font highlights significantly higher values in Fisher exact test, adjusted *P* value <0.05). Proteins were grouped according to their functions (GO terms).

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

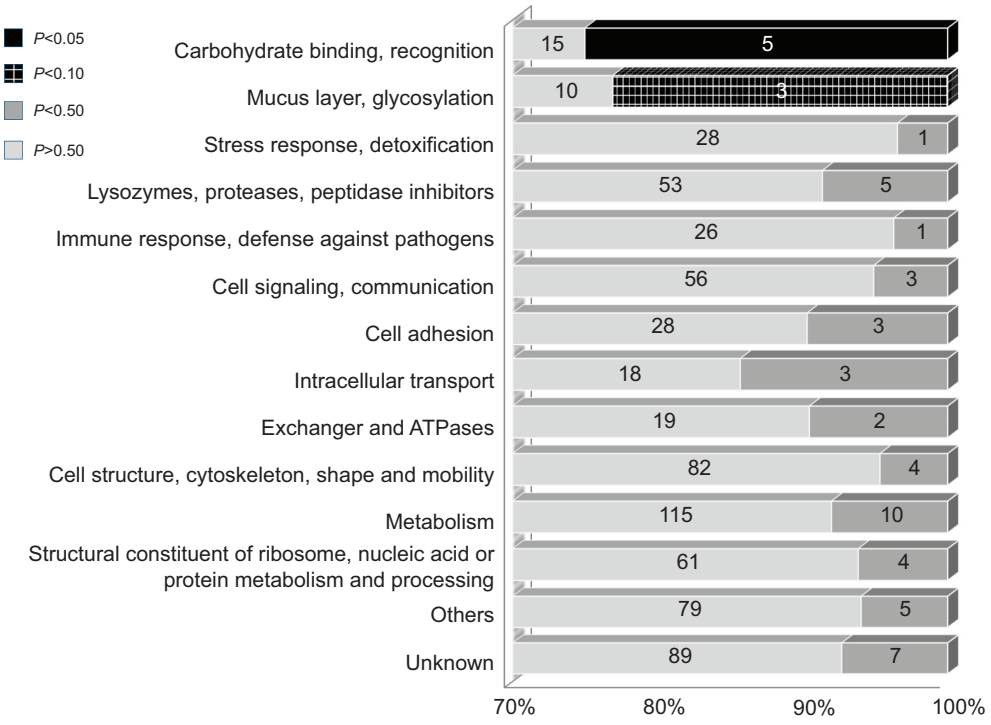

Fig. 3. Gene ontology (GO) terms identified from the enrichment analysis. Only the two categories identified as 'Carbohydrate binding, recognition' and 'Mucus layer, glycosylation' include proteins significantly enriched in the dorsal and ventral tract, respectively (Fisher's exact test, $P<0.05$). Numbers on the bars represent the number of proteins in each category, and are reflected in the percentage values displayed on the *x*-axis.

(Table S3). The overall standardized spectral count of this group of 20 proteins in the dorsal tract (368) was more than double that in the ventral tract (168) and the abundance of 16 of these proteins was significantly higher in the dorsal tract as compared to the ventral tract. These included three C-type lectins: C-type lectin 2-like protein and two C-type mannose receptors 2 (Table 2). Among the C-type lectins found in the dorsal tract (Table S3), the three C-type mannose receptors 2 (cds.c102634_g1_i1|m.3912/CvML3912; Pales Espinosa and Allam, 2018; cds.c102634_g1_i2|m.3914; cds.c102634_g1_i3|m.3915) present highly similar protein sequences (e.g. carbohydrate recognition domain and determinant motifs for calcium binding and sugar specificity).

In order to better understand why these C-type lectins are more abundant in the dorsal tract, the precise localization of the cells that produce the CvML3912 mRNA and cognate protein, *in situ* hybridization (ISH) was performed on oyster gills and results are presented in Fig. 4. Positive cells were recorded along the different types of gill filaments (Fig. 1C,E) and an average number was calculated for the apical zone (apical ordinary filaments, 15.6±0.73) and the lateral zone (i.e. lateral ordinary and principal filaments, 19.5±0.79). The results indicate a higher number of positive cells on the lateral zone of the plicae as compared with the apical zone, supporting the enrichment of the fluid mucus present in the dorsal tract with CvML3912, and possibly other isoforms or closely related C-type lectins.

A main function of gills in suspension-feeding bivalves (e.g. oyster *C. virginica*), in addition to respiration, is the capture and transport of food particles (Atkins, 1936, 1937; Galtsoff, 1964; Ribelin and Collier, 1977). Using elaborate ciliary mechanisms, particles captured on gills are directed either to a dorsal tract (i.e. basal ciliated tract, Fig. 1) or to a ventral tract (i.e. marginal ciliated groove). More specifically, particles reaching the principal filaments of the gills are carried to the dorsal tract while particles attaining the ordinary filaments, and most specifically the apical ordinary filaments, are either directed to the ventral tract in the counter-current created by cilia beating ventrally [Fig. 1E, frontal median tract (FMT); Beninger et al., 2005; Beninger and StJean, 1997] or to the dorsal tract in the current created by cilia beating dorsally [Fig. 1E, two frontal lateral tracts (FLTs); Beninger et al., 2005; Ribelin and Collier, 1977], but in this last case, always via the principal filaments (Ward et al., 1994). Overall, most particles traveling via the ventral tract are fated for rejection in pseudofeces while particles trapped in the dorsal tract are directed to the mouth via the palps (where secondary sorting can occur) to be ingested, even though this mechanism is also dependent on particle concentration, particle size and on the satiation status of the bivalves (Beninger et al., 1992; Cognie et al., 2003; Ward et al., 1994). Regardless of the path the particles follow, their transport is enabled by the presence of mucus. In metazoans, mucus is often three-dimensionally structured with the presence of two distinct layers covering epithelial cells (Ross and Corrsin, 1974). The inner layer is in direct contact with the epithelial cells and is often made of low viscosity mucus that allows cilia beating. The outer layer is typically made of non-continuous and viscous secretions that entrap particles and is directed by cilia movements. This two-layer model has been confirmed in bivalves (Beninger et al., 1997), and is particularly relevant along the rejection pathway (i.e. ventral tract) where particles are embedded in viscous rafts of mucus floating on a low-viscosity mucus. This viscous mucus (enriched in acid mucopolysaccharides) is mainly produced by a high number of mucocytes lining the epithelium of the apical ordinary filaments (Beninger and Dufour, 1996; Beninger and StJean, 1997). Among the molecules known to affect mucus viscosity (Girod et al., 1992), the mucin/mucin-like proteins, their relative concentrations, and the degree of their glycosylation and hydration have been found to be the most critical (Cone, 2009; Lai et al., 2009; Linden et al., 2008). For example, the viscosity of a gel made of mucins from the giant West African snail (*Archachatina marginata*; Momoh et al., 2019) or from the coral (*Montastrea faveolata*; Jatkar et al., 2010) increases with the increase in mucin concentration. Our results showing a high abundance of mucin-like proteins and other proteins with adhesive properties (e.g. proteins with VWA and EGF domains) in the ventral tract are in agreement with earlier studies reporting a

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

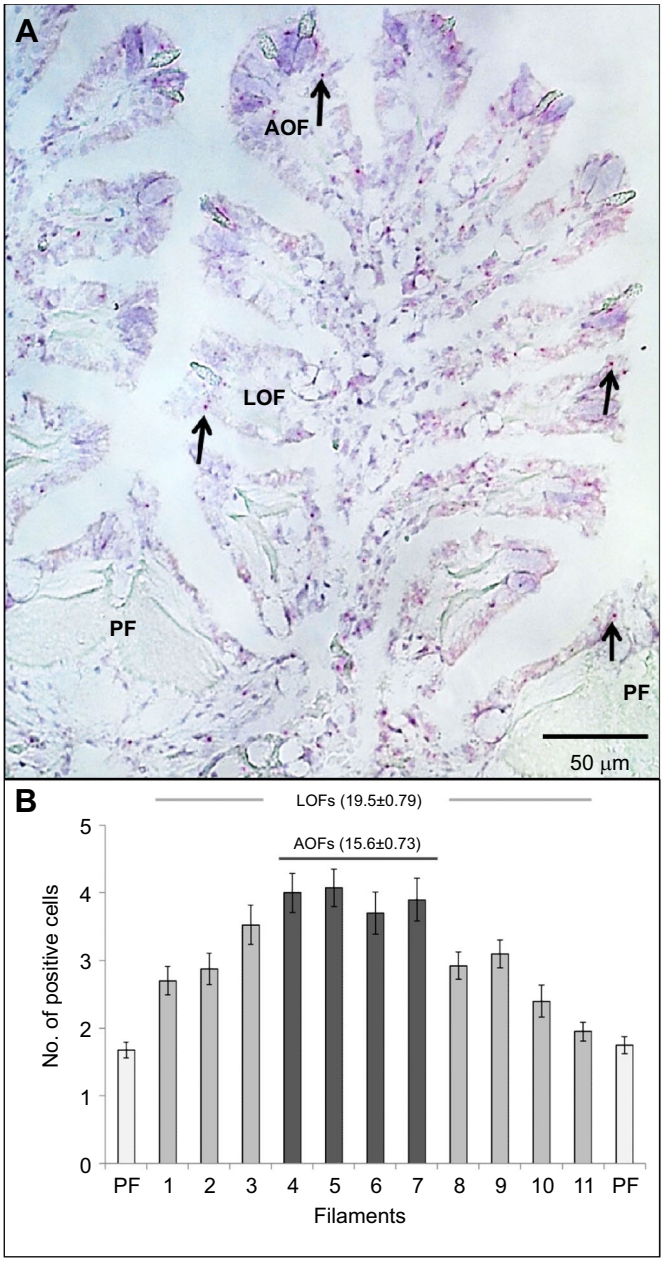

Fig. 4. *In situ* hybridization localization of *CvML3912* transcripts on the gill plicae of *Crassostrea virginica*. (A) Transverse section of gill plica. Black arrows indicate positive cells on apical ordinary filaments (AOF), lateral ordinary filaments (LOF) and principal filaments (PF). Scale bar: 50 µm. (B) Counts of positive cells from *in situ* hybridization. Means±s.e. are presented for each filament (*n*=90 plicae).

higher viscosity of this mucus as compared to mucus from the dorsal tract. Even though the exact roles of the VWA and EGF domains are not well known, several studies highlighted the fact that they may cross-link components of the mucus, therefore increasing mucus adhesion (Li and Graham, 2007; Smith et al., 2017).

The ventral tract is an area particularly exposed to water turbulence and currents and therefore the high viscosity of mucus present in this area helps trap particles aimed for rejection (Beninger and Dufour, 1996). It is noteworthy that the ventral tract was enriched in galactose- and fucose-binding lectins (adhesive properties), suggesting that particles having cell surfaces rich in galactose or fucose residues may preferentially bind to mucus present in this rejection tract. These results corroborate our previous findings showing that microalgae with cell surfaces rich in galactose-related carbohydrates are preferentially rejected in pseudofeces (Pales Espinosa et al., 2016a).

In contrast, the mucus present in the dorsal tract (i.e. ingestion pathway) originates from the principal filament troughs and the lateral ordinary filaments (Fig. 1C), both described as having a low density in mucocytes (Beninger and Dufour, 1996). This mucus, as discussed above, is characterized by a low abundance of mucin-like proteins and displays a lower viscosity than the mucus from the ventral tract (Beninger and Dufour, 1996; Beninger and StJean, 1997). It can easily flow toward the plicae trough (i.e. principal filament) driven by water currents that enter oyster pallial cavity in a ventrodorsal orientation and are deflected laterally towards the plical troughs (Fig. 1D). This low viscosity mucus carries a multitude of molecules produced by the filament cells into the plical troughs (above the principal filament) while avoiding the dispersal and loss of these molecules in the surrounding seawater. If the mucus from the dorsal tract is less viscous as compared to the ventral mucus, its adhesive property seems to be high and specific. These characteristics are likely due to the presence of numerous C-type lectins and most specifically several ‘C-type mannose receptors 2’, whose affinity for mannose/glucose residues was demonstrated for at least one of them (i.e. cds.c102634_g1_i1|m.3912; Pales Espinosa and Allam, 2018). Our results showed that this lectin is produced by the cells of the principal and ordinary filaments and is, on average, more abundant in the area made by the lateral ordinary filaments (Fig. 4). mRNAs of several C-type lectins have already been reported in the epithelium of the pallial organs and digestive tract of different bivalve species (Pales Espinosa et al., 2010; Yamaura et al., 2008) and further proteomic analysis of *C. virginica* confirmed that some of these cognate proteins were secreted into the pallial mucus (Pales Espinosa et al., 2016b). This new high spatial resolution analysis allowed the detection of additional lectins, including CvML, a C-type lectin previously suspected to be secreted into mucus (i.e. C-type lectin 1; Jing et al., 2011; Table S3). These findings support a high specificity of mucus from the dorsal tract to a certain type of ligand. In particular, the marked enrichment of mannose/glucose-binding lectins in the dorsal tract (i.e. ingestion pathway) is in line with previous results showing preferential ingestion of microalgae having mannose/glucose on their cell surface (Pales Espinosa et al., 2016a).

Altogether, the results presented in this study, in conjunction with previous information (Beninger et al., 2005; Pales Espinosa and Allam, 2018; Pales Espinosa et al., 2016a), provide a fine-scale mechanistic explanation for the particle selection process in oysters and likely other suspension-feeding bivalves (Fig. 5). Particles (e.g. microalgae, unicellular parasites, debris) not predominantly covered with mannose/glucose residues are more likely to be trapped by the thick mucus present at the apex of the plicae, possibly via adhesion proteins, and directed to the ventral tract to be rejected in pseudofeces. By contrast, particles covered with mannose and glucose residues are more likely to be bound by mannose/glucose-binding lectins (e.g. C-type mannose receptors 2) present abundantly in the fluid mucus that flows along the plical troughs and would be then directed to the dorsal tract for ingestion.

Microbial neutralization and digestion

Pallial mucus in bivalves is involved in the processing of an extraordinarily large number of waterborne microbes that enter the pallial cavity. Some of these microbes will serve as food as described above but others may be harmful for the health of these animals. The role of mucus in host–microbe interactions and animal protection is now well recognized across various taxa and has

Journal of Experimental Biology (2021) 224, jeb233361. doi:10.1242/jeb.233361

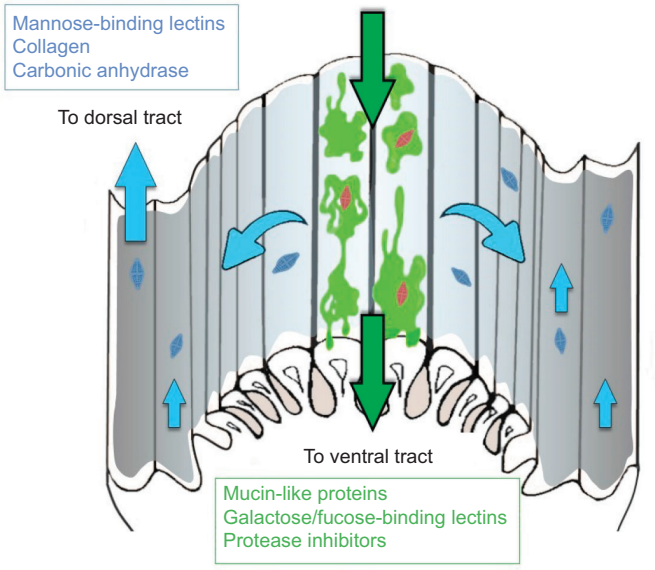

Fig. 5. Schematic representation of mucus movement and particle transport on a typical plica of the gill of the oyster *Crassostrea virginica*. Most functionally relevant proteins from the two mucosal tracts are listed.

gained prominence in the past few decades as a main component of the innate and acquired immune system (Allam and Pales Espinosa, 2015; Russell et al., 2015). In *C. virginica*, mucosal secretions are not only an excellent physical barrier but also contain host defensive cells (Lau et al., 2017) and a multitude of bioactive compounds (Pales Espinosa et al., 2016b) that act against microbe proliferation.

In this study, the analysis of both ventral and dorsal tracts revealed the presence of numerous proteins involved in host-microbe interactions, and more specifically, in defense against pathogens. For example, several proteins regrouped under 'lysozymes, proteases and peptidase inhibitors' were more abundant in the ventral tract (Table S3). This is for example the case of 3 peptidase inhibitors (kazal-type serine proteinase inhibitor, metalloproteinase inhibitor 1-like and pancreatic trypsin inhibitor) that were significantly more abundant in the ventral tract as compared to the dorsal tract (Table 2). Protease inhibitors regulate the activity of peptidases (Rawlings et al., 2004) and are considered to represent determinant resistance factors against infectious diseases in mollusks by preventing the harmful activity of exogenous proteases produced by invading microorganisms (La Peyre et al., 2010; Xue et al., 2006; Yu et al., 2011).

By contrast, other proteins linked to defense against pathogens were more abundant in the dorsal tract as compared to the ventral tract (Table S3). This is, for example, the case for three 'peptidoglycan recognition proteins' (i.e. PGRPs) and two lysozymes (cds.c101519_g1_i1|m.3174, cds.c113002_g1_i1|m.21368) known to have a bacteriolytic role in several bivalves (Maginot et al., 1989; Su et al., 2007), including oysters (Cronin et al., 2001; Itoh and Takahashi, 2008; Xue et al., 2010). Similarly, the abundance of the 'complement c1q tumor necrosis factor-related protein 3' (i.e. C1q-TNF, cds.c110839_g1_i1|m.15938), several 'complement C1q-like' (e.g. cds.c114572_g2_i1|m.25743), a 'β-glucan-binding protein' (i.e. BGBP, cds.c122006_g1_i2|m.52775) and two 'peptidases' (kyphoscoliosis peptidase and tolloid-like protein 2 harboring a trypsin domain, Table 2) were found to be significantly higher in the dorsal tract. In higher vertebrates, C1q-TNF family (e.g. CTRP3) is thought to mediate a large number of biological processes, including inflammation and glucose homeostasis (Li et al., 2011). In bivalves, this family of proteins is considered as an essential contributor to non-self recognition and immunity (Gestal et al., 2010). Similarly, the BGBP family is well known to play a significant role in invertebrate immunity (Vargas-Albores and Yepiz-Plascencia, 2000), including in bivalves (Liu et al., 2014). Furthermore, the abundance of the glycosyl hydrolase 'β-galactosidase-1-like protein' (cds.c122884_g1_i1|m.56982; Table 2), which is known to catalyze the hydrolysis of galactosides into monosaccharides, was also found to be significantly higher in the dorsal as compared to the ventral tract. β-galactosidases are produced by hemocytes (Moore and Gelder, 1985) as well as the secreting cells (i.e. apocrine cells) located in the epithelium of the digestive tract (e.g. esophagus, intestine) of mollusks (Martin et al., 2011). They actively participate in the intracellular digestion of microbes after phagocytosis (Moore and Gelder, 1985). This enzyme could contribute to the early digestion of microbes although its source in the dorsal tract is unknown but might be related to a possible higher abundance of specific secretory cells along the lateral ordinary filaments and/or principal filaments of the gill plicae.

Proteins with unknown functions

A difference between the ventral and dorsal tracts was also found for the abundance of proteins whose functions in bivalve mollusks are not well defined, making the interpretation of the findings tentative (Table 2). This was the case, for example, for two proteins involved in intracellular transport ('sodium-coupled neutral amino acid transporter partial' and 'sodium-dependent phosphate transport protein 2b', also called NaPi2B; Table 2) that were significantly more abundant in the ventral tract as compared to the dorsal tract. In humans, neutral amino acid transporters (e.g. SLC1A5) are suggested to play an important role in amino acid depletion in mucus that cover lungs in order to deprive pathogenic organisms from their nutrients, limiting their propagation (Mager and Sloan, 2003). In vertebrates, NaPi2b has been suggested to play a role in the synthesis of surfactant in lung alveoli (Hashimoto et al., 2000) whose main role is to facilitate respiration. A similar role in facilitating gas exchange between gill cells and their environment could be suggested for this protein in bivalves.

As another example, two proteins involved in the 'chitin metabolic process' (peritrophin-1-like and uncharacterized protein loc101848577) were significantly more abundant in the ventral tract as compared to the dorsal (Table 2). Chitin is known to play an important role in shell formation in oysters (Suzuki et al., 2007; Zhang et al., 2012) even though this complex mechanism is not well understood. In addition, some peritrophins from insects possess one or several highly glycosylated mucin-like domains (Hegedus et al., 2016; Wang and Granados, 1997), which may contribute to mucus viscosity and possibly explain their presence in the mucus from the ventral tract.

Several collagen proteins (cell structure, cytoskeleton, shape and mobility) were more abundant in the dorsal (225 spectral counts; Table S3) as compared to the ventral tract (62 spectral counts) with the abundance of two of these (collagen alpha-6 chain, cds.c113381_g1_i1|m.22332 and cds.c114415_g2_i1|m.25328; Table 2) being significantly higher. In animals, collagen alpha-6 chain protein is the major structural component of the basement membrane. In bivalve gills, collagen is also present in the pair of skeletal rods that strengthen all types of filaments (Galtsoff, 1964; Le Pennec et al., 1988) and in muscle tissues (Medler and Silverman, 1998). The presence of these proteins in mucus from the dorsal tract can be explained by the large size of the skeleton rods and the position of the interlamellar septa (muscular tissue), both located beneath the principal filaments in close proximity to this tract.

The dorsal tract was also significantly enriched with 'carbonic anhydrase 2-like' (Table 2). Carbonic anhydrases are enzymes that

catalyze the formation of hydrogen carbonate (HCO_3^-) from carbon dioxide (CO_2) and water (Khalifah, 1971). In mollusks, these molecules were suggested to play a major role in acid–base balance (Wang et al., 2017), mediating the accumulation of calcium in mantle and gill tissue, and enabling the biomineralization process (Cudennec et al., 2006; Miyamoto et al., 1996). It has also been proposed that carbonic anhydrases are involved in ion regulation processes (osmoregulation) by generating HCO_3^- that can serve as counter-ions in sodium (Na^+) and potassium (K^+) uptake (Henry and Saintsing, 1983; Hu et al., 2011). While carbonic anhydrases have been found abundantly in the gills of bivalves (Duvail et al., 1998; Henry and Saintsing, 1983), it remains unclear why this protein is more abundant in the dorsal tract.

Conclusions

This fine-scale analysis of mucus revealed major proteomic differences between the dorsal and ventral tracts of the gill of *C. virginica* and suggests that each of these tracts upholds functional specialization, including their precise role in particle transport. Results showed that the dorsal tract (transport of particles intended for ingestion) is enriched with mannose- and glucose-binding lectins, providing a mechanistic explanation of previous experimental findings showing preferential ingestion of microalgae with cell surfaces covered with mannose and glucose residues (Pales Espinosa et al., 2016a). In parallel, the enrichment of mucin-like molecules and other adhesive proteins in the ventral tract is in line with prior studies showing a high viscosity of mucus in this tract. Overall, the results demonstrate that the molecular signature of mucus in each tract is different and can be linked to their specific function. However, the lack of information about the function of some proteins limits our ability to generate a complete picture of the functional topography of oyster gills. Additional studies should evaluate the effective ability of mucus from each tract to differentially interact with waterborne microbes.

Acknowledgements

We thank Drs E. Ward and M. Rosa (technical assistance with oyster and mucus collection), Dr D. Martin (support for proteomic analysis) and Drs E. Corre and S. Bassim (bioinformatics support).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.P.E., B.A.; Methodology: E.P.E., B.A.; Validation: E.P.E., B.A.; Formal analysis: E.P.E., B.A.; Investigation: E.P.E., B.A.; Resources: E.P.E., B.A.; Data curation: E.P.E., B.A.; Writing - original draft: E.P.E., B.A.; Writing - review & editing: E.P.E., B.A.; Visualization: E.P.E., B.A.; Supervision: E.P.E., B.A.; Project administration: E.P.E., B.A.; Funding acquisition: E.P.E., B.A.

Funding

This work was supported by grants from the National Science Foundation (IOS 1050596 and IOS 1146920).

Supplementary information

Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.233361.supplemental

References

Allen, W. R. (1921). Studies of the biology of freshwater mussels - Experimental studies of the food relations of the Unionidae. *Biol. Bull.* **40**, 210-241. doi:10.2307/1536464

Allam, B. and Pales Espinosa, E. (2015). Mucosal immunity in mollusks. In *Mucosal Health in Aquaculture* (ed. B. Beck and E. Peatman), pp. 325-370: Academic Press.

Allam, B., Carden, W. E., Ward, J. E., Ralph, G., Winnicki, S. and Pales Espinosa, E. (2013). Early host-pathogen interactions in marine bivalves: Evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces. *J. Invertebr. Pathol.* **113**, 26-34. doi:10.1016/j.jip.2012.12.011

Atkins, D. (1936). Memoirs: on the ciliary mechanisms and interrelationships of lamellibranchs: Part I: new observations on sorting mechanisms. *J. Cell Sci.* **2**, 181-308.

Atkins, D. (1937). On the ciliary mechanisms and interrelationships of lamellibranchs. Part II: Sorting devices on the gills. *Q. J. Microsc. Sci.* **79**, 339-373.

Barille, L. and Cognie, B. (2000). Revival capacity of diatoms in bivalve pseudofaeces and faeces. *Diatom. Res.* **15**, 11-17. doi:10.1080/0269249X.2000.9705483

Bayne, B. L., Iglesias, J. I. P., Hawkins, A. J. S., Navarro, E., Heral, M. and Deslouspaoli, J. M. (1993). Feeding behavior of the mussel *Mytilus edulis*: responses to variations in quantity and organic content of the seston. *J. Mar. Biol. Assoc. U. K.* **73**, 813-829. doi:10.1017/S0025315400034743

Beninger, P. and Dufour, S. (1996). Mucocyte distribution and relationship to particle transport on the pseudolamellibranch gill of *Crassostrea virginica* (Bivalvia: Ostreidae). *Mar. Ecol. Prog. Ser.* **137**, 133-138. doi:10.3354/meps137133

Beninger, P. G. and StJean, S. D. (1997). The role of mucus in particle processing by suspension-feeding marine bivalves: unifying principles. *Mar. Biol.* **129**, 389-397. doi:10.1007/s002270050179

Beninger, P. G., Ward, J. E., Macdonald, B. A. and Thompson, R. J. (1992). Gill function and particle-transport in *Placopecten magellanicus* (mollusca, bivalvia) as revealed using video endoscopy. *Mar. Biol.* **114**, 281-288. doi:10.1007/BF00349531

Beninger, P. G., St-Jean, S., Poussart, Y. and Ward, J. E. (1993). Gill function and mucocyte distribution in *Placopecten magellanicus* and *Mytilus edulis* (Mollusca, Bivalvia) - the role of mucus in particle-transport. *Mar. Ecol. Prog. Ser.* **98**, 275-282. doi:10.3354/meps098275

Beninger, P. G., Lynn, J. W., Dietz, T. H. and Silverman, H. (1997). Mucociliary transport in living tissue: The two-layer model confirmed in the mussel *Mytilus edulis* L. *Biol. Bull.* **193**, 4-7. doi:10.2307/1542730

Beninger, P. G., Cannuel, R. and Jaunet, S. (2005). Particle processing on the gill plicae of the oyster *Crassostrea gigas*: fine-scale mucocyte distribution and functional correlates. *Mar. Ecol. Prog. Ser.* **295**, 191-199. doi:10.3354/meps295191

Burreson, E. M. and Ford, S. E. (2004). A review of recent information on the *Haplosporidia*, with special reference to *Haplosporidium nelsoni* (MSX disease). *Aquat. Living Resour.* **17**, 499-517. doi:10.1051/alr:2004056

Caruana, N. J., Cooke, I. R., Faou, P., Finn, J., Hall, N. E., Norman, M., Pineda, S. S. and Strugnell, J. M. (2016). A combined proteomic and transcriptomic analysis of slime secreted by the southern bottletail squid, Sepiadarium austrinum (Cephalopoda). *J. Proteomics* **148**, 170-182. doi:10.1016/j.jprot.2016.07.026

Cognie, B., Barillé, L. and Rincé, E. (2001). Selective feeding of the oyster *Crassostrea gigas* fed on a natural microphytobenthos assemblage. *Estuaries* **24**, 126-134. doi:10.2307/1352819

Cognie, B., Barillé, L., Massé, G. and Beninger, P. G. (2003). Selection and processing of large suspended algae in the oyster Crassostrea gigas. *Mar. Ecol. Prog. Ser.* **250**, 145-152. doi:10.3354/meps250145

Cone, R. A. (2009). Barrier properties of mucus. *Adv. Drug Delivery. Rev.* **61**, 75-85. doi:10.1016/j.addr.2008.09.008

Cronin, M. A., Culloty, S. C. and Mulcahy, M. F. (2001). Lysozyme activity and protein concentration in the haemolymph of the flat oyster Ostrea edulis (L.). *Fish Shellfish Immunol.* **11**, 611-622. doi:10.1006/fsim.2001.0338

Cudennec, B., Rousseau, M., Lopez, E. and Fouchereau-Peron, M. (2006). CGRP stimulates gill carbonic anhydrase activity in molluscs via a common CT/CGRP receptor. *Peptides* **27**, 2678-2682. doi:10.1016/j.peptides.2006.05.019

Dahl, S., Thiel, J. and Allam, B. (2010). QPX disease progress in cultured and wild type hard clams in New York waters. *J. Shellfish Res.* **29**, 83-90. doi:10.2983/035.029.0131

Davies, M. S. and Hawkins, S. J. (1998). Mucus from marine molluscs. In *Advances in Marine Biology*, Vol. 34 (ed. J. H. S. Blaxter, A. J. Southward and P. A. Tyler), pp. 1-71. London: Academic Press Ltd-Elsevier Science Ltd.

Denny, M. W. (1989). Invertebrate mucous secretions: functional alternatives to vertebrate paradigms. In *Symposia of the Society for Experimental Biology, XLIII. Mucus and Related Topics* (ed. E. Chantler and N. A. Ratcliffe), pp. 337-366. Cambridge The Company of Biologists Limited.

Dubilier, N., Bergin, C. and Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat. Rev. Microbiol.* **6**, 725-740. doi:10.1038/nrmicro1992

Dufour, S. C. (2005). Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae. *Biol. Bull.* **208**, 200-212. doi:10.2307/3593152

Duvail, L., Moal, J. and Fouchereau-Peron, M. (1998). CGRP-like molecules and carbonic anhydrase activity during the growth of Pecten maximus. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **120**, 475-480. doi:10.1016/S0742-8413(98)10068-3

Galtsoff, P. S. (1964). The American oyster *Crassostrea virginica* Gmelin. *Fish. Bull.* **64**, 1-480.

Gatlin, C. L., Kleemann, G. R., Hays, L. G., Link, A. J. and Yates, J. R. (1998). Protein identification at the low femtomole level from silver-stained gels using a

new fritless electrospray interface for liquid chromatography–microspray and nanospray mass spectrometry. *Anal. Biochem.* **263**, 93-101. doi:10.1006/abio.1998.2809

Gavagnin, M., Spinella, A., Castelluccio, F., Cimino, G. and Marin, A. (1994). Polypropionates from the Mediterranean mollusk *Elysia timida*. *J. Nat. Prod.* **57**, 298-304. doi:10.1021/np50104a017

Gestal, C., Pallavicini, A., Venier, P., Novoa, B. and Figueras, A. (2010). MgC1q, a novel C1q-domain-containing protein involved in the immune response of *Mytilus galloprovincialis*. *Dev. Comp. Immunol.* **34**, 926-934. doi:10.1016/j.dci.2010.02.012

Girod, S., Zahm, J., Plotkowski, C., Beck, G. and Puchelle, E. (1992). Role of the physiochemical properties of mucus in the protection of the respiratory epithelium. *Eur. Respir. J.* **5**, 477-487.

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talón, M., Dopazo, J. and Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* **36**, 3420-3435. doi:10.1093/nar/gkn176

Gustafson, K. and Andersen, R. J. (1985). Chemical studies of British Columbia nudibranchs. *Tetrahedron* **41**, 1101-1108. doi:10.1016/S0040-4020(01)96478-6

Hargens, A. R. and Shabica, S. V. (1973). Protection against lethal freezing temperatures by mucus in an Antarctic limpet. *Cryobiology* **10**, 331-337. doi:10.1016/0011-2240(73)90052-7

Hashimoto, M., Wang, D.-Y., Kamo, T., Zhu, Y., Tsujiuchi, T., Konishi, Y., Tanaka, M. and Sugimura, H. (2000). Isolation and localization of type IIb Na/Pi cotransporter in the developing rat lung. *Am. J. Pathol.* **157**, 21-27. doi:10.1016/S0002-9440(10)64512-9

Hegedus, D. D., Toprak, U. and Erlandson, M. (2016). Lepidopteran peritrophic matrix composition, function, and formation. In *Short Views on Insect Genomics and Proteomics*, pp. 63-87: Springer.

Henry, R. P. and Saintsing, D. G. (1983). Carbonic anhydrase activity and ion regulation in three species of osmoregulating bivalve molluscs. *Physiol. Zool.* **56**, 274-280. doi:10.1086/physzool.56.2.30156059

Hu, M. Y., Tseng, Y.-C., Lin, L.-Y., Chen, P.-Y., Charmantier-Daures, M., Hwang, P.-P. and Melzner, F. (2011). New insights into ion regulation of cephalopod molluscs: a role of epidermal ionocytes in acid-base regulation during embryogenesis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R1700-R1709. doi:10.1152/ajpregu.00107.2011

Itoh, N. and Takahashi, K. G. (2008). Distribution of multiple peptidoglycan recognition proteins in the tissues of Pacific oyster, *Crassostrea gigas*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **150**, 409-417. doi:10.1016/j.cbpb.2008.04.011

Itoh, N., Xue, Q.-G., Schey, K. L., Li, Y., Cooper, R. K. and La Peyre, J. (2011). Characterization of the major plasma protein of the eastern oyster, Crassostrea virginica, and a proposed role in host defense. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **158**, 9-22. doi:10.1016/j.cbpb.2010.06.006

Jatkar, A. A., Brown, B. E., Bythell, J. C., Guppy, R., Morris, N. J. and Pearson, J. P. (2010). Coral mucus: the properties of its constituent mucins. *Biomacromolecules* **11**, 883-888. doi:10.1021/bm9012106

Jing, X., Pales Espinosa, E., Perrigault, M. and Allam, B. (2011). Identification, molecular characterization and expression analysis of a mucosal C-type lectin in the eastern oyster, *Crassostrea virginica*. *Fish Shellfish Immunol.* **30**, 851-858. doi:10.1016/j.fsi.2011.01.007

Khalifah, R. G. (1971). The carbon dioxide hydration activity of carbonic anhydrase I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **246**, 2561-2573. doi:10.1016/S0021-9258(18)62326-9

Kremer, N., Philipp, E. E. R., Carpentier, M.-C., Brennan, C. A., Kraemer, L., Altura, M. A., Augustin, R., Häsler, R., Heath-Heckman, E. A. C., Peyer, S. M. et al. (2013). Initial symbiont contact orchestrates host-organ-wide transcriptional changes that prime tissue colonization. *Cell Host Microbe* **14**, 183-194. doi:10.1016/j.chom.2013.07.006

Lai, S. K., Wang, Y.-Y., Wirtz, D., Hanes, J. (2009). Micro-and macrorheology of mucus. *Adv. Drug Delivery. Rev.* **61**, 86-100. doi:10.1016/j.addr.2008.09.012

La Peyre, J. F., Xue, Q.-G., Itoh, N., Li, Y. and Cooper, R. K. (2010). Serine protease inhibitor cvSI-1 potential role in the eastern oyster host defense against the protozoan parasite *Perkinsus marinus*. *Dev. Comp. Immunol.* **34**, 84-92. doi:10.1016/j.dci.2009.08.007

Lau, Y.-T., Sussman, L., Pales Espinosa, E., Katalay, S. and Allam, B. (2017). Characterization of hemocytes from different body fluids of the eastern oyster Crassostrea virginica. *Fish Shellfish Immunol.* **71**, 372-379. doi:10.1016/j.fsi.2017.10.025

Le Pennec, M., Beninger, P. and Herry, A. (1988). New observations of the gills of Placopecten magellanicus (Mollusca: Bivalvia), and implications for nutrition. *Mar. Biol.* **98**, 229-237. doi:10.1007/BF00391199

Li, D. M. and Graham, L. D. (2007). Epiphragmin, the major protein of epiphragm mucus from the vineyard snail, *Cernuella virgata*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **148**, 192-200. doi:10.1016/j.cbpb.2007.05.009

Li, Y., Wright, G. L. and Peterson, J. M. (2011). C1q/TNF–related protein 3 (CTRP3) function and regulation. *Comp. Physiol.* **7**, 863-878. doi:10.1002/cphy.c160044

Linden, S., Sutton, P., Karlsson, N., Korolik, V. and McGuckin, M. (2008). Mucins in the mucosal barrier to infection. *Mucosal Immunol.* **1**, 183-197. doi:10.1038/mi.2008.5

Liu, S., Qi, Z., Zhang, J., He, C., Gao, X. and Li, H. (2014). Lipopolysaccharide and β-1, 3-glucan binding protein in the hard clam (*Meretrix meretrix*): Molecular characterization and expression analysis. *Genet. Mol. Res.* **13**, 4956. doi:10.4238/2014.July.4.10

Mager, S. and Sloan, J. (2003). Possible role of amino acids, peptides, and sugar transporter in protein removal and innate lung defense. *Eur. J. Pharmacol.* **479**, 263-267. doi:10.1016/j.ejphar.2003.08.075

Maginot, N., Samain, J. F., Daniel, J. Y., Le Coz, J. R. and Moal, J. (1989). Kinetic properties of lysozyme from the digestive glands of Ruditapes philippinarum. *Oceanis* **15**, 451-464.

Mann, K. and Jackson, D. J. (2014). Characterization of the pigmented shell-forming proteome of the common grove snail Cepaea nemoralis. *BMC Genomics* **15**, 249. doi:10.1186/1471-2164-15-249

Marie, B., Joubert, C., Tayalé, A., Zanella-Cléon, I., Belliard, C., Piquemal, D., Cochennec-Laureau, N., Marin, F., Gueguen, Y. and Montagnani, C. (2012). Different secretory repertoires control the biomineralization processes of prism and nacre deposition of the pearl oyster shell. *Proc. Natl Acad. Sci. USA* **109**, 20986-20991. doi:10.1073/pnas.1210552109

Martin, G. G., Martin, A., Tsai, W. and Hafner, J. C. (2011). Production of digestive enzymes along the gut of the giant keyhole limpet Megathura crenulata (Mollusca: Vetigastropoda). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **160**, 365-373. doi:10.1016/j.cbpa.2011.07.003

McDowell, I. C., Nikapitiya, C., Aguiar, D., Lane, C. E., Istrail, S. and Gomez-Chiarri, M. (2014). Transcriptome of American oysters, *Crassostrea virginica*, in response to bacterial challenge: insights into potential mechanisms of disease resistance. *PLoS ONE* **9**, e105097. doi:10.1371/journal.pone.0105097

Medler, S. and Silverman, H. (1998). Extracellular matrix and muscle fibers in the gills of freshwater bivalves. *Invertebr. Biol.* **117**, 288-298. doi:10.2307/3227031

Miyamoto, H., Miyashita, T., Okushima, M., Nakano, S., Morita, T. and Matsushiro, A. (1996). A carbonic anhydrase from the nacreous layer in oyster pearls. *Proc. Natl Acad. Sci. USA* **93**, 9657-9660. doi:10.1073/pnas.93.18.9657

Momoh, M. A., Chime, S. A., Ogbodo, D. U., Akudike, P. K., Udochukwu, S. U., Ossai, E. C., Kenechukwu, F. C., Ofokansi, K. C. and Attama, A. A. (2019). Biochemical, rheological and hydrophile-lipophile balance (HLB) evaluation of Archachatina marginata (snail) mucin extract for possible nutraceutical and nano biopharmaceutical applications. *Trop. J. Pharm. Res.* **18**, 927-934. doi:10.4314/tjpr.v18i5.3

Moore, C. and Gelder, S. (1985). Demonstration of lysosomal enzymes in hemocytes of Mercenaria mercenaria (Mollusca: Bivalvia). *Trans. Am. Microsc. Soc.* **104**, 242-249. doi:10.2307/3226436

Morton, B. (1977). The hypobranchial gland in the Bivalvia. *Can. J. Zool.* **55**, 1225-1234. doi:10.1139/z77-161

Nesvizhskii, A. I., Keller, A., Kolker, E. and Aebersold, R. (2003). A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **75**, 4646-4658. doi:10.1021/ac0341261

Newell, R. I. E. and Jordan, S. J. (1983). Preferential ingestion of organic material by the american oyster *Crassostrea virginica*. *Mar. Ecol. Prog. Ser.* **13**, 47-53. doi:10.3354/meps013047

Pales Espinosa, E. and Allam, B. (2018). Reverse genetics demonstrate the role of mucosal C-type lectins in food particle selection in the oyster Crassostrea virginica. *J. Exp. Biol.* **221**, jeb174094. doi:10.1242/jeb.174094

Pales Espinosa, E., Perrigault, M., Ward, J. E., Shumway, S. E. and Allam, B. (2009). Lectins associated with the feeding organs of the oyster, *Crassostrea virginica*, can mediate particle selection. *Biological Bulletin* **217**, 130-141. doi:10.1086/BBLv217n2p130

Pales Espinosa, E., Perrigault, M. and Allam, B. (2010). Identification and molecular characterization of a mucosal lectin (MeML) from the blue mussel *Mytilus edulis* and its potential role in particle capture. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **156**, 495-501. doi:10.1016/j.cbpa.2010.04.004

Pales Espinosa, E., Winnicki, S. M. and Allam, B. (2013). Early host-pathogen interactions in marine bivalves: Pallial mucus of *Crassostrea virginica* modulates the growth and virulence of its pathogen *Perkinsus marinus*. *Dis. Aquat. Org.* **104**, 237-247. doi:10.3354/dao02599

Pales Espinosa, E., Corre, E. and Allam, B. (2014). Pallial mucus of the oyster *Crassostrea virginica* regulates the expression of putative virulence genes of its pathogen *Perkinsus marinus*. *Int. J. Parasitol.* **44**, 305-317. doi:10.1016/j.ijpara.2014.01.006

Pales Espinosa, E., Cerrato, R. M., Wikfors, G. and Allam, B. (2016a). Modeling food choice in suspension-feeding bivalves. *Mar. Biol.* **163**, 2-13. doi:10.1007/s00227-016-2815-0

Pales Espinosa, E., Koller, A. and Allam, B. (2016b). Proteomic characterization of mucosal secretions in the eastern oyster, *Crassostrea virginica*. *J. Proteomics* **132**, 63-76. doi:10.1016/j.jprot.2015.11.018

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**, 346-356. doi:10.1016/0003-2697(77)90043-4

Prezant, R. S. and Chalermwat, K. (1984). Flotation of the bivalve *Corbicula fluminea* as a means of dispersal. *Science* **225**, 1491-1493. doi:10.1126/science.225.4669.1491
Rawlings, N. D., Tolle, D. P. and Barrett, A. J. (2004). Evolutionary families of peptidase inhibitors. *Biochem. J.* **378**, 705-716. doi:10.1042/bj20031825
Ribelin, B. W. and Collier, A. (1977). Studies on the gill ciliation of the American oyster *Crassostrea virginica* (Gmelin). *J. Morphol.* **151**, 439-449. doi:10.1002/jmor.1051510308
Riisgard, H. U., Larsen, P. S. and Nielsen, N. F. (1996). Particle capture in the mussel *Mytilus edulis*: The role of latero-frontal cirri. *Mar. Biol.* **127**, 259-266. doi:10.1007/BF00942111
Rose, M. C., Voter, W. A., Brown, C. F. and Kaufman, B. (1984). Structural features of human tracheobronchial mucus glycoprotein. *Biochem. J.* **222**, 371-377. doi:10.1042/bj2220371
Ross, S. and Corrsin, S. (1974). Results of an analytical model of mucociliary pumping. *J. Appl. Physiol.* **37**, 333-340. doi:10.1152/jappl.1974.37.3.333
Roxas, B. A. P. and Li, Q. (2008). Significance analysis of microarray for relative quantitation of LC/MS data in proteomics. *BMC Bioinformatics* **9**, 187. doi:10.1186/1471-2105-9-187
Russell, M. W., Mestecky, J., Strober, W., Lambrecht, B. N., Kelsall, B. L. and Cheroutre, H. (2015). Overview: The Mucosal Immune System. *Mucosal Immunol.* **1**, 3-8. doi:10.1016/B978-0-12-415847-4.00001-X
Schachter, H. and Williams, D. (1982). Biosynthesis of mucus glycoproteins. In *Mucus in Health and Disease—II*, Vol. 144 (ed. E. Chantler, J. Elder and M. Elstein), pp. 3-28: Springer US.
Simkiss, K. and Wilbur, K. M. (1977). The molluscan epidermis and its secretions. *Symp. Zool. Soc. Lond.* **39**, 35-76.
Smith, A. M. (2002). The structure and function of adhesive gels from invertebrates. *Integr. Comp. Biol.* **42**, 1164-1171. doi:10.1093/icb/42.6.1164
Smith, A. M. and Morin, M. C. (2002). Biochemical differences between trail mucus and adhesive mucus from marsh periwinkle snails. *Biol. Bull.* **203**, 338-346. doi:10.2307/1543576
Smith, A. M., Quick, T. J. and Peter, R. S. (1999). Differences in the composition of adhesive and non-adhesive mucus from the limpet Lottia limatula. *Biol. Bull.* **196**, 34-44. doi:10.2307/1543164
Smith, A. M., Papaleo, C., Reid, C. W. and Bliss, J. M. (2017). RNA-Seq reveals a central role for lectin, C1q and von Willebrand factor A domains in the defensive glue of a terrestrial slug. *Biofouling* **33**, 741-754. doi:10.1080/08927014.2017.1361413
Southward, E. C. (1986). Gill symbionts in Thyasirids and other bivalve molluscs. *J. Mar. Biol. Assoc. U. K.* **66**, 889-914. doi:10.1017/S0025315400048517
Su, J. G., Ni, D. J., Song, L. S., Zhao, H. M. and Qiu, L. M. (2007). Molecular cloning and characterization of a short type peptidoglycan recognition protein (CfPGRP-S1) cDNA from Zhikong scallop *Chlamys farreri*. *Fish Shellfish Immunol.* **23**, 646-656. doi:10.1016/j.fsi.2007.01.023
Suzuki, M., Sakuda, S. and Nagasawa, H. (2007). Identification of chitin in the prismatic layer of the shell and a chitin synthase gene from the Japanese pearl oyster, Pinctada fucata. *Biosci. Biotechnol. Biochem.* **71**, 1735-1744. doi:10.1271/bbb.70140
Urrutia, M. B., Navarro, E., Ibarrola, I. and Iglesias, J. I. P. (2001). Preingestive selection processes in the cockle *Cerastoderma edule*: mucus production related to rejection of pseudofaeces. *Mar. Ecol. Prog. Ser.* **209**, 177-187. doi:10.3354/meps209177
Vargas-Albores, F. and Yepiz-Plascencia, G. (2000). Beta glucan binding protein and its role in shrimp immune response. *Aquaculture* **191**, 13-21. doi:10.1016/S0044-8486(00)00416-6
Wang, P. and Granados, R. R. (1997). Molecular cloning and sequencing of a novel invertebrate intestinal mucin cDNA. *J. Biol. Chem.* **272**, 16663-16669. doi:10.1074/jbc.272.26.16663
Ward, J. E., Beninger, P. G., Macdonald, B. A. and Thompson, R. J. (1991). Direct observations of feeding structures and mechanisms in bivalve mollusks using endoscopic examination and video image-analysis. *Mar. Biol.* **111**, 287-291. doi:10.1007/BF01319711
Ward, J. E., Macdonald, B. A., Thompson, R. J. and Beninger, P. G. (1993). Mechanisms of suspension-feeding in bivalves - resolution of current controversies by means of endoscopy. *Limnol. Oceanogr.* **38**, 265-272. doi:10.4319/lo.1993.38.2.0265
Ward, J. E., Newell, R. I., Thompson, R. J. and MacDonald, B. A. (1994). *In vivo* studies of suspension-feeding processes in the eastern oyster, *Crassostrea virginica* (Gmelin). *Biol. Bull.* **186**, 221-240. doi:10.2307/1542056
Ward, J. E., Levinton, J. S., Shumway, S. E. and Cucci, T. (1998). Particle sorting in bivalves: in vivo determination of the pallial organs of selection. *Mar. Biol.* **131**, 283-292. doi:10.1007/s002270050321
Wang, F., Flanagan, J., Su, N., Wang, L.-C., Bui, S., Nielson, A., Wu, X., Vo, H.-T., Ma, X.-J. and Luo, Y. (2012). RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagn.* **14**, 22-29. doi:10.1016/j.jmoldx.2011.08.002
Wang, X., Wang, M., Jia, Z., Qiu, L., Wang, L., Zhang, A. and Song, L. (2017). A carbonic anhydrase serves as an important acid-base regulator in pacific oyster Crassostrea gigas exposed to elevated CO 2: implication for physiological responses of mollusk to ocean acidification. *Mar. Biotechnol.* **19**, 22-35. doi:10.1007/s10126-017-9734-z
Washburn, M. P., Wolters, D. and Yates, J. R. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **19**, 242-247. doi:10.1038/85686
Wenger, C. D., Phanstiel, D. H., Lee, M., Bailey, D. J. and Coon, J. J. (2011). COMPASS: a suite of pre–and post–search proteomics software tools for OMSSA. *Proteomics* **11**, 1064-1074. doi:10.1002/pmic.201000616
Wolcott, T. G. (1973). Physiological ecology and intertidal zonation in limpets (Acmaea): a critical look at" limiting factors". *Biol. Bull.* **145**, 389-422. doi:10.2307/1540048
Xue, Q.-G., Waldrop, G. L., Schey, K. L., Itoh, N., Ogawa, M., Cooper, R. K., Losso, J. N. and La Peyre, J. F. (2006). A novel slow-tight binding serine protease inhibitor from eastern oyster *Crassostrea virginica* plasma inhibits perkinsin, the major extracellular protease of the oyster protozoan parasite *Perkinsus marinus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **145**, 16-26. doi:10.1016/j.cbpb.2006.05.010
Xue, Q., Hellberg, M. E., Schey, K. L., Itoh, N., Eytan, R. I., Cooper, R. K. and La Peyre, J. F. (2010). A new lysozyme from the eastern oyster, *Crassostrea virginica*, and a possible evolutionary pathway for i-type lysozymes in bivalves from host defense to digestion. *BMC Evol. Biol.* **10**, 213. doi:10.1186/1471-2148-10-213
Xue, Q., Gauthier, J., Schey, K., Li, Y., Cooper, R., Anderson, R. and La Peyre, J. (2012). Identification of a novel metal binding protein, segon, in plasma of the eastern oyster, *Crassostrea virginica*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **163**, 74-85. doi:10.1016/j.cbpb.2012.05.002
Xue, Q., Beguel, J.-P. and La Peyre, J. (2019). Dominin and segon form multiprotein particles in the plasma of Eastern Oysters (Crassostrea virginica) and are likely involved in shell formation. *Front. Physiol.* **10**, 566. doi:10.3389/fphys.2019.00566
Yamaura, K., Takahashi, K. G. and Suzuki, T. (2008). Identification and tissue expression analysis of C-type lectin and galectin in the Pacific oyster, *Crassostrea gigas*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **149**, 168-175. doi:10.1016/j.cbpb.2007.09.004
Yu, H., He, Y., Wang, X., Zhang, Q., Bao, Z. and Guo, X. (2011). Polymorphism in a serine protease inhibitor gene and its association with disease resistance in the eastern oyster (*Crassostrea virginica* Gmelin). *Fish Shellfish Immunol.* **30**, 757-762. doi:10.1016/j.fsi.2010.12.015
Zhang, G. F., Fang, X. D., Guo, X. M., Li, L., Luo, R. B., Xu, F., Yang, P. C., Zhang, L. L., Wang, X. T., Qi, H. G. et al. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* **490**, 49-54. doi:10.1038/nature11413