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Immune response of the Antarctic bivalve *Laternula elliptica* to physical stress and microbial exposure

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ABSTRACT

Marine bivalves, such as the Antarctic soft shell clam *Laternula elliptica* (King and Broderip 1831), experience a wide range of environmental influences including the permanent contact to the surrounding microbial community due to filter feeding or strong physiochemical disturbances in a changing environment. Such impacts are anticipated to influence *L. elliptica* physiology, including the immune system, especially under the current climate change conditions at the Western Antarctic Peninsula (WAP). To understand basal immune defence reactions in *L. elliptica*, we investigated *in vitro* hemocyte responses upon bacterial challenge or after stimulation with different substances of microbial origin (pathogen associated molecular patterns, PAMPS). To study the age-dependent *in vivo* response of hemocyte abundance, starvation and injury experiments were undertaken with young and old individuals. Hemocytes of *L. elliptica* feature common immune functions such as phagocytosis and ROS generation, as well as agglutination of bacterial cells. ROS generation response to different stimuli was generally low and lower in larger/older animals compared to smaller/younger individuals. Physiological conditions such as size/age, starvation and injury modulated the abundance of hemocytes in *L. elliptica*. Implications are that current and future climate change conditions in West Antarctic coastal ecosystems scenarios may lead to changes in species survival and population composition.

1. Introduction

The Antarctic soft shell clam *Laternula elliptica* is a major biomass component in the muddy sediments of the maritime Antarctic and also a key species in the Antarctic bentho-pelagic carbon flux of shallow coastal areas (Ahn, 1993; Momo et al., 2002). As filter-feeders, these bivalves ingest sedimentary particles and planktonic organisms from the sediment–water interface. In bivalves, bacteria, free-living or associated to dead or living particles, are ingested and can be highly concentrated on the gill surfaces and in the digestive system (Prieur et al., 1990; Pruzzo et al., 2005). These bacteria can be either a source of the bivalve's nutrition, become part of the microflora in a commensal or symbiontic association with the host, or act as potentially harmful invaders (Pruzzo et al., 2005). Under conditions affecting the bivalves' physiological state (e.g. starvation) or during bacterial mass-proliferations due to eutrophication or warming, pathogenic bacteria were found to cause mass mortalities in different bivalve species (Cheney et al., 2000; Cao et al., 2007; Garnier et al., 2007; Allen and Burnett, 2008). Molluscs have evolved immune mechanisms for the recognition, immobilization and clearance of invading microorganisms. Components of an adaptive immune system are lacking in bivalves (Bachere et al., 1995; Canesi et al., 2002) and recognition of microorganisms is mediated by plasma proteins, glycoproteins and circulating hemocyte cells. Hemocyte receptors can bind directly or via mediating "adapter-molecules" (opsonization) on surface molecules which are present on the outer membranes of microorganisms. Following recognition of nonself particles by the molluscan immune system, humoral and cellular processes are initiated to kill and eliminate the invaders (Canesi et al., 2002). The humoral response includes permanent or inducible biosynthesis of antimicrobial peptides or enzymes (Bettencourt et al., 2007). Cellular reactions such as phagocytosis or encapsulation of particles are mediated by hemocytes (Cheng, 1975; Malham et al., 2003).

A main part of the invertebrate and vertebrate immunocyte response consists in the generation of reactive oxygen species (ROS) "respiratory burst", triggered upon contact of immune cells with the microorganisms. Catalyzed by a membrane bound NAD(P)H-oxidase (NOX), molecular oxygen is reduced to superoxide anion which generates various highly reactive oxygen metabolites (hydrogen peroxides, singlet oxygen or hydroxyl radicals) (Lambeth, 2004). Oxidative burst-like reactions have been recorded in several bivalves (Anderson, 1994; Boyd and Burnett, 1999; Donaghy et al., 2009) and involve the production of

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superoxide ($O_2^{\bullet}$) and/or nitric oxides (NO) under control conditions and after stimulation of hemocytes with microbial mimetics (Arumugam et al., 2000; Bettencourt et al., 2007). The main types of bivalve hemocytes comprise granula containing granulocytes and cells with low amounts or no granules (hyalinocytes), with the granulocytes generally exhibiting a higher ROS generation potential compared to hyalinocytes (Hegaret et al., 2003; Labreuche et al., 2006a; Labreuche et al., 2006b). In the present study we provide a first characterization of hemocytes of the Antarctic bivalve *L. elliptica* from Potter Cove, King-George Island, West Antarctica, and tested the response to different substances mimicking bacterial infections. The study was conducted in respect to the slow but steady warming, changing ice dynamics, and the increasing coastal run-off in the West Antarctic Peninsula region (Kloeser et al., 1994; Varela, 1998; Dierssen et al., 2002). Animals colonizing the shallow areas around the South Shetland Islands, such as *L. elliptica*, are bound to be the first to suffer the impact of this change. Increased physical disturbances due to intensified iceberg scouring (Smale and Barnes, 2008) as well as changing food quality and quantity due to increased inflow of inorganic particles in coastal areas, are anticipated to influence *L. elliptica* physiology (Philipp et al., in press) and could also compromise hemocyte immune function. The ongoing rapid climate change in the West Antarctic Peninsula region might render animals from stress-exposed coastal environments more susceptible to bacterial infections, and not only further changing temperature, light, pH and salinity regimes and eutrophication, but also enhanced Antarctic tourism may affect bacterial species composition and potentially introduce more harmful species into the coastal environment (Paz et al., 2007; Garcia et al., 2009; Turner et al., 2009). Filter feeder key species like *L. elliptica* might therefore be applicable models to study early effects of environmental changes at the organism level.

Specifically, we characterized the antibacterial capacity of *L. ellip*tica tissues and hemolymph as well as hemocyte morphology and ability of phagocytosis. The respiratory burst response of *L. elliptica* hemocytes was studied after *in vitro* stimulation with environmental bacteria from the Potter Cove environment, and with well known microbial stimuli (LPS, PMA, flagellin, zymosan, peptidoglycan and ODN), using the nitro blue tetrazolium chloride (NBT) test. Hemocyte response to stimulation with microbial mimetics or cultivated bacteria was further microscopically investigated and described. Starvation and injury experiments, mimicking damage due to iceberg scour, were undertaken with small and large *L. elliptica*, to record changes in hemocyte abundance of young and old individuals under such stress situations.

2. Material and methods

2.1. Sampling of animals

L. elliptica were collected by divers in Potter Cove, King George Island (62°14′S, 58°40′W), Antarctic Peninsula, between November 2008 and February 2009 at 10–15 m depth, 33.5 PSU and 1.5 °C. Animals were classified as "large" with (mean and S.D.) 7.22 ± 0.52 cm and "small" with 4.77 ± 0.47 cm shell length. At the Dallmann Laboratory on the Argentinean Jubany Station, animals were kept in natural seawater at constant temperature of 1 °C for at least one week. Every second day 50% of the water in the holding system was renewed to ensure good water quality. Following acclimation, hemolymph was sampled from 50 individuals for hemocyte characterization (morphology, phagocytosis, and ROS generation). Further 163 individuals were used for hemocyte counts (HC) in distinctly sized/aged *L. elliptica* individuals exposed to different stress scenarios.

2.2. Hemolymph collection and hemocyte counts

Hemolymph fluid containing the hemocyte cells was collected by inserting a 16-gauge needle with a 10 ml-syringe into the posterior adductor muscle and slowly withdrawing the hemolymphatic fluid. Between 3 and 6 ml of hemolymph was harvested per animal.

For HC, hemolymph samples were diluted (1:10) with ice-cold modified Alsever's anticoagulant solution (NaCl 22.5 g l^{-1}, Glucose 20.8 g l^{-1}, Sodium citrate 8 g l^{-1}, ethylene-tetraacetic acid (EDTA) 3.36 g l^{-1} in deionized and sterile-filtered (0.2 μm) water), to avoid hemocyte aggregation (Bachere, et al., 1988). Hemocytes were counted twice in a hemocytometer (Neubauer improved counting chamber) and densities calculated as hemocytes per ml. Undiluted samples of single individuals were used for stimulation and phagocytosis experiments to avoid allogenic reactions which may influence hemocyte reactions.

2.3. General hemocyte characterization

2.3.1. Hemocyte morphology

Freshly collected hemolymph was applied on cleaned cooled glass slides, and hemocytes were allowed to spread out on the surface before covering the cells with a coverslip. To investigate phagocytic activity, hemocytes were incubated with yeast cells that had previously been stained with bromphenol blue. Hemocyte morphology was observed by light microscopy.

2.3.2. Characterization of hemocyte immune response

To stimulate hemocytes, different pathogen associated molecular patterns (PAMPs) were applied. Lipopolysaccharide (LPS, research center Borstel) and peptidoglycan (PGN, BioChemika 77140) are important structural constituents of the outer membrane of gram-negative (LPS and PGN) and gram-positive (PGN) bacteria.

Phorbol-12-myristate-13-acetate (PMA, BioChemika 79352) is a toxic cell proliferation promoter in vertebrates. Flagellin (Invivogen), a main component of bacterial flagellar filament, was derived from *Salmonella typhimurium* and is a stimulator of epithelial surface defense responses in different eukaryotic cell lines. Zymosan (Sigma Z-4250), a protein–carbohydrate-complex, is derived from yeast cell walls, and widely used as experimental inducer of inflammation or phagocytosis. Synthetic oligonucleotides (ODN2006 — type B, Invivogen), with incorporated specific unmethylated dinucleotide sequences can act as immunostimulants or proliferation inducers. Concentrations of stimulants were chosen as recommended by the manufacturers or other studies and stock solutions were prepared as follows: LPS [1 mg/ml], PMA [20 μM], flagellin [2 μg/ml], zymosan [10 mg/ml], peptidoglycan [200 μg/ml], and ODN [50 μM]. Many of these molecules are strongly toxic (endotoxins) and, therefore, represent potential stimulants of the immune systems of vertebrates and invertebrates.

To stimulate hemocytes with natural bacteria, environmental bacteria were isolated from Potter Cove seawater, as well as from the animals and their closest sedimentary environment. Two *L. elliptica* individuals with surrounding sediment and seawater were collected by divers into a glass flask. The flask was kept at constant temperature (1 °C) for several hours. Subsequently, animals were removed and released to the sea. After complete sedimentation in the flask, the overlying water was decanted and filtered through a cellulose acetate membrane (0.22 μm pore size). Seawater samples were collected from 10 m water depth in Potter Cove and also filtered over 0.22 μm pore size filters. Each filter was placed into a test tube containing 10 ml of one-tenth-strength nutrient broth (Merck), using filtered seawater instead of distilled water for dilution, and cultured at 10 °C for two days. Bacteria were collected by centrifugation (2300 *g*, 15 min, 4 °C) and resuspended in sterile filtered seawater. Both living and dead (heat-killed) bacteria were used for hemocyte stimulation.

2.3.3. Microscopic observations of the immune response

Aliquots of 70 μl of individual hemolymph samples were placed directly onto cooled, cleaned microscope glass slides. After 10 min the

different PAMPS or bacteria (20 µl) were added to the attached hemocytes. Sterile seawater substituted the stimulating reagent in controls. The cells were covered with a coverslip. After 15–30 min incubation in cooled humid chambers (4 °C), hemocytes were observed by light microscopy (Zeiss Axioskop) with 40× and 100× magnification. Documentation was done by digital imagery. At least triplicate experiments were run for all substances of microbial origin, each with hemolymph of a single individual.

2.3.4. Effect of bacterial mimetics on the oxidative burst of L. elliptica hemocytes: NBT reduction assay

The production of ROS by hemocyte cells was measured by the nitroblue terazolium (NBT)-reduction assay after Anderson et al. (1992) and Arumugam et al. (2000). The assay measures $O_2^{\bullet-}$, NO and presumably other ROS such as peroxynitrite (NOO•) and quantifies NBT-formazan production resulting from the interaction of ROS with NBT (Anderson et al., 1992; Arumugam et al., 2000; Bettencourt et al., 2007). For each experiment, a 500 µl aliquot of hemolymph from one individual was placed in sterile 1.5 ml-reaction tubes on ice. As it was shown that some hemolymph factors seem to be important for ROS generation (Kumazawa et al., 1993), serum was not replaced by a buffer-system. Only hemolymph samples with >700,000 cells per ml were used for these investigations, to assure sufficient sensitivity of the test-system. Samples were gently mixed with 50 µl of stimulant solution (microbial mimetic) or sterile seawater for controls and with 550 µl NBT solution (0.1% in sterile seawater, 0.2 µm filtered and kept on ice). Stimulated and control tubes were incubated for 60 min at 3 °C in the dark and inverted every 15 min to ensure mixing. After incubation, the samples were centrifuged (400 *g*, 2 °C, 5 min), supernatants discarded and pellets re-suspended in 550 µl methanol (70%). After a second centrifugation (400 *g*, 2 °C, 5 min) supernatants were discarded again and pellets re-suspended in 350 µl extraction solution (6 ml of 2 M KOH and 7 ml DMSO) by vigorous shaking on a vortex mixer to dissolve the formazan. After an additional centrifugation step (3500 *g*, 8 °C, 20 min) to remove cellular debris, the optical density (OD) of the supernatant was recorded at 630 nm in triplicates of 100 µl in a 96-well-microtiterplate versus an extraction solution blank using a TRISTAR multiplate reader (Fa. Berthold, Germany). Results were normalized to hemocyte number in the sample aliquot.

As zymosan exposure resulted in the highest ROS generation response, this substance was applied in an age dependent test of hemocyte oxidative burst response. Hemolymph samples of large and small specimens (n = 5–6) were individually collected as described above and subdivided into 3 aliquots. Two were mixed with zymosan (50 µl, 10 mg/ml) and one with the corresponding volume of sterile seawater. NBT was added and samples were incubated and processed as described above.

2.3.5. Antibacterial assays

To investigate whether *L. elliptica* tissue and hemolymph possess antibacterial properties, two different antibacterial assays were performed. Seawater bacteria and bacteria from the sedimentary environment of *L. elliptica* were plated on halfstrength marine agar plates and incubated at 10 °C until growth was observed. Colonies displaying different morphotypes were re-suspended separately to obtain pure cultures. For assay (A), homogenates of *L. elliptica* gills (200 mg/ml) were prepared in phosphate buffered saline (PBS) containing a protease inhibitor (Aprotinin, 1 µg/ml). Gill homogenates and pure hemolymph samples were pooled from 5 animals, applied onto sterile filter strips and placed on marine agar plates that had previously been streaked with lines of isolated bacterial morphotypes. In total 25 bacterial isolates were tested and control experiments run with sterile seawater instead of the gill homogenate or hemolymph. After 3 days of incubation, areas around the strips were checked for zones of inhibited bacterial growth.

For assay (B), 10 µl of bacteria in suspension was mixed with samples of pooled hemolymph or gill homogenates and controls (seawater) and plated onto marine agar. After 12 h of incubation colony counts were performed.

2.4. Whole animal experiments

To check, whether environmental stress alters immune functions of *L. elliptica*, small and large animals were collected and subjected to starvation, high nutrient loads and injury. Animals were kept in 4 separate tanks (150 l) with filtered seawater (10 µm final pore size) without sediment and maintained at a constant temperature of 1 °C and fully aerated. After 10 days of acclimation the experiment was started: half of the animals were subjected to filtered seawater (0.2 µm final pore size, treatment "starvation") and the other half to unfiltered seawater enriched with dissolved and particulate nutrients (treatment "food"). The mixed diet consisted of live microalgae as well as "artificial detritus": freeze dried and pestled macroalgae

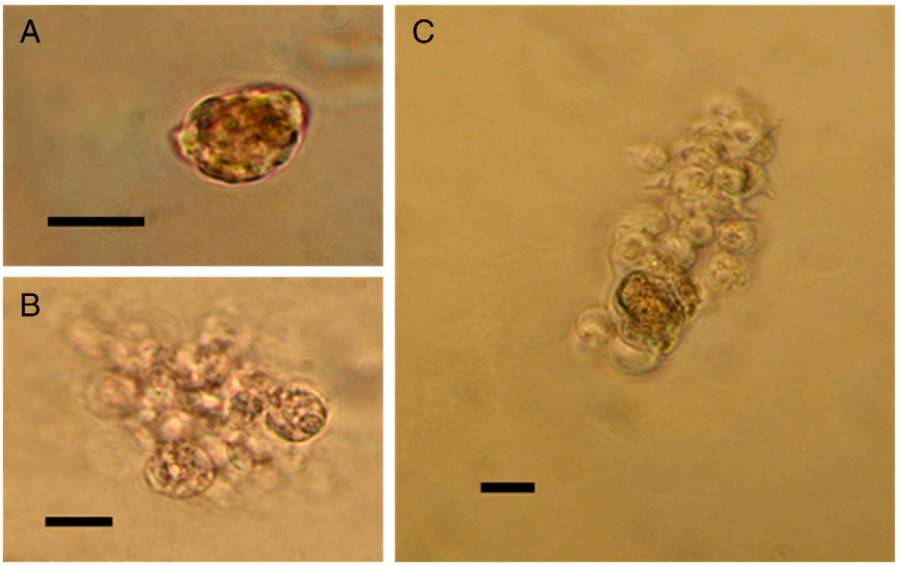

Fig. 1. Hemocytes of *L. elliptica*: (A) optically dense, larger cell type 1; (B) spherical hemocyte, transparent and with less or without granula and inclusions (cell type 2); (C) both cell types forming cluster. Scale bar = 10 µm.

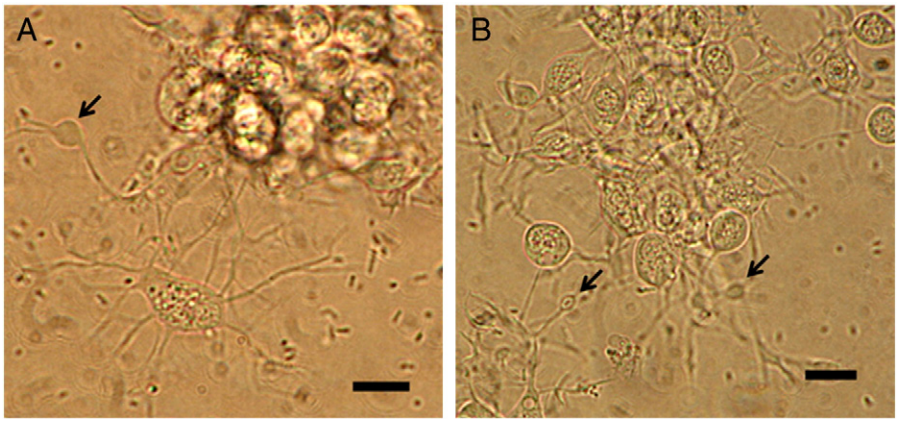

Fig. 2. Hemocytes of *L. elliptica*: Fresh preparations, unfixed. Hemocytes formed aggregates (A) and single cells, mostly of the less granular type 2, spread out on the microscopic slide generating filopodia (A and B) with visible swellings due to plasma flow (arrows). Scale bar = 10 μm.

from Potter Cove, as well as freeze dried and pestled krill and red bloodworms (Tetra, Melle, Germany). Microalgae were obtained from Potter Cove and kept in culture under light in 2 l-bottles. Animals were fed every second day.

After 21 days 50% of the animals from both treatments were injured artificially. Both valves of the animals were cracked by a soft blow with a blunt tool (wrench) in such a way that also the mantle was injured. Additionally the siphon was cut at two places. After the injury event, the animals were allowed to recover in their tank of origin under the appropriate nutrition condition. Hemolymph for hemocyte counts (HC) of animals from the different treatments was sampled in 10 days acclimated animals (controls, n = 7–10), at day 23 after start of the experiment i.e. for the "injury group" two days after the injury event, and at day 44, after 21 days of regeneration from the injury, without sampling any individual twice (n = 5–12). After the hemolymph collection, animals were sacrificed and tissues frozen for analysis.

2.4.1. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software). Data were tested for normality by the Kolmogorov–Smirnov test. Differences between two groups were evaluated by Student t-tests. Analysis of variance (ANOVA) was used to look for differences of hemocyte numbers in life animal experiment among the injury, starvation and food groups compared to the control individuals.

3. Results

3.1. Hemocyte description

The hemolymph of *L. elliptica* contains two major types of free circulating hemocytes. A small fraction of less than 10% of the whole *L. elliptica* hemocyte population has numerous opaque granules and inclusions (cell type 1, Fig. 1A). Hemocytes of this type range between 12 and 16 μm in diameter, and their shape is often asymmetrical. These cells appeared optically rather dense and generally darker than the major cell type (cell type 2, >90%) which was of spherical shape and contained few or no transparent granules. Type two cells varied in size between 7 and 13 μm in diameter and contained a well visible round nucleus (Fig. 1B). These cells quickly formed numerous and branched extensions (filopodia) within minutes after plating on microscopic slides (Fig. 2).

Streaming of protoplasma and transport of particles or vacuolic inclusions were observed in the filopodia. Plasma and particle flow caused swellings of the tips of the filopodia (Fig. 2A, B; arrows). Some hemocytes were of fusiform (spindle-shaped) appearance (Fig. 2A). Granular cells (type 1) did not form many filopodia but aggregated with the second, more abundant cell type 2.

Spontaneous aggregate formation of hemocytes in suspension or on microscopic slides in untreated or undiluted hemolymph was always observed. Approximately 5 min after attachment, the cells began to spread, associate and connect via an actively growing "net" of filopodia (Fig. 2). Non-attached aggregates were composed

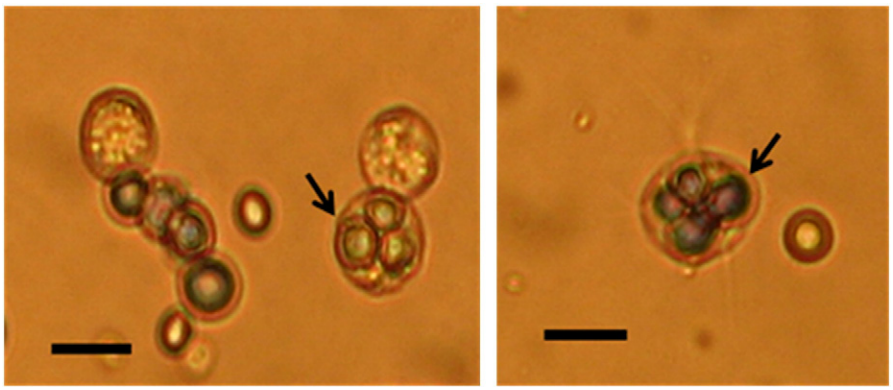

Fig. 3. Hemocytes of *L. elliptica*. Hemolymph was mixed and incubated with yeast cells (stained with bromphenol blue). Some hemocytes showed phagocytic activity and engulfed yeast cells (arrows). Scale bar = 10 μm.

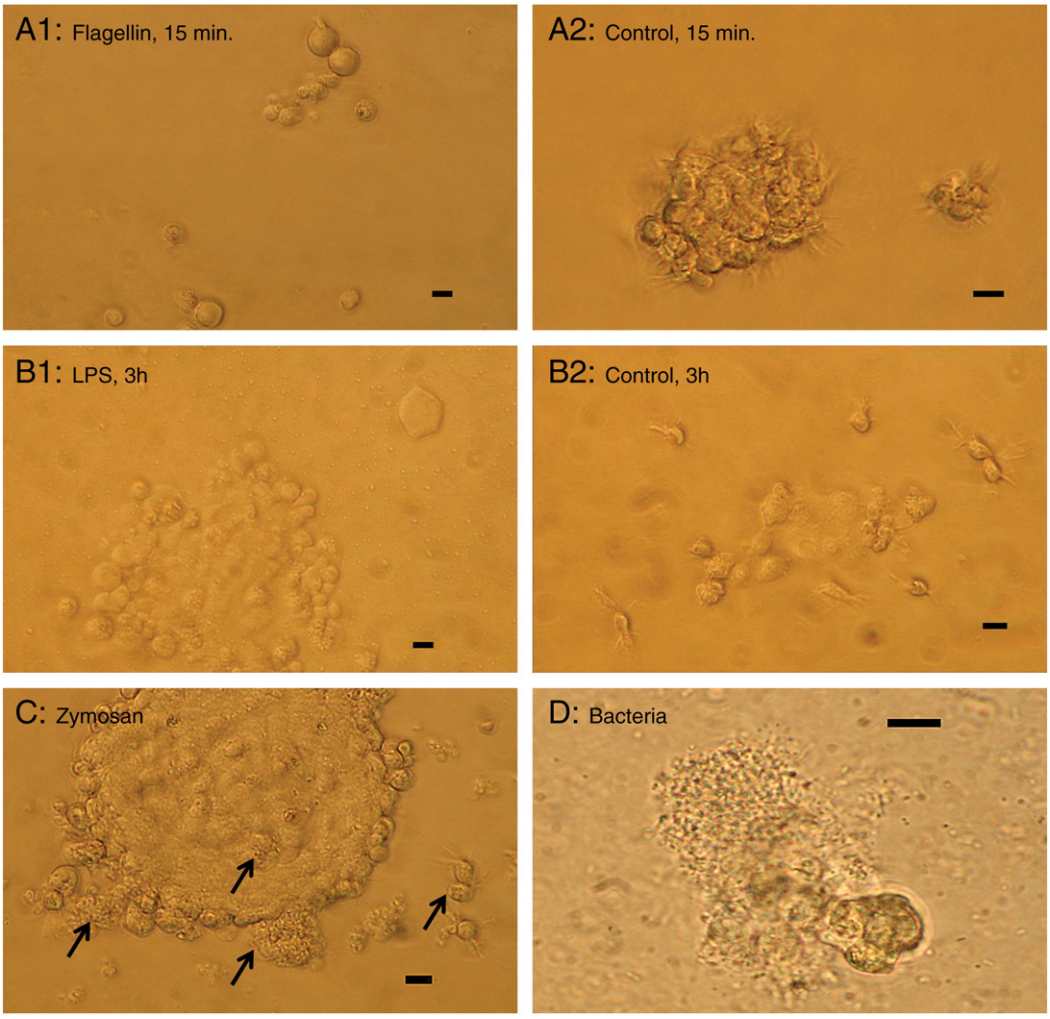

Fig. 4. Hemocytes of *L. elliptica*. Hemolymph incubated with different microbial mimetics and with bacteria isolated from Potter Cove seawater and sediments. (A1) Incubation with flagellin (15 min): less aggregates, less extension of filopodia than seawater controls (A2, 15 min). (B1) Incubation with lipopolysaccharide (LPS, 3 h): beginning of cell lysis. (B2) Control incubation (seawater, 3 h): extension of filopodia, formation of aggregates, no cell lysis. (C) Incubation with zymosan: zymosan particles aggregated and attached to hemocytes (arrows). (D) Hemocytes mixed with bacteria: bacteria were adsorbed to hemocyte exudates. Scale bar = 10 μm.

of sometimes several hundreds of cells, in which differently sized hemocytes of "less-granular" morphotypes and small amounts of dark granular cells were included. Cells in these aggregates developed shorter filopodia extending towards the outside medium. Type 2 hemocytes were capable of phagocyting heat killed yeast cells (Fig. 3). After 30 min of incubation, single hemocytes or small aggregates of hemocytes were observed that contained yeast cells, whereas other cells were in close contact or aggregated with yeast cells (Fig. 3). This was not observed for the granular type 1 hemocytes.

3.2. Hemocyte stimulation experiments

The presence of flagellin (15 min incubation) affected aggregation of hemocytes (Fig. 4A1). Most of the cells floated freely in suspension and a lesser amount attached to the microscope slides, spread out and aggregated when compared to the seawater treated controls (Fig. 4A2). Some cells appeared "swollen" with less formation of filopodia compared to controls. Treatment (15 min) of hemocytes with LPS, PMA, peptidoglycan and ODN did not have a visible effect on aggregate formation. After 3 h of incubation cells under LPS treatment started to lyse, membranes disintegrated, (Fig. 4B1) whereas cells in controls remained intact (Fig. 4B2). Particles of zymosan were agglutinated and aggregates were bound to the surface of aggregating hemocytes. In some cells, phagocyted particles of zymosan were visible (Fig. 4C). Living and heat-killed bacteria were immobilized and adsorbed by the hemocyte aggregates (Fig. 4D). These effects were observed in treatments with both natural bacterial isolates and with both types of hemocytes.

3.3. ROS production by stimulated hemocytes

A baseline level of ROS production was always observed in unstimulated hemolymph samples (Tables 1 and 2, Fig. 6). Addition of Flagellin, PGN, PMA, Zymosan and ODN to the hemocytes induced

Table 1
Generation of ROS in the hemolymph of *L. elliptica* measured as formazan formation. PGN: peptidoglycan, LPS: lipopolysaccharide, PMA: phorbol-12-myristate-13-acetate, ODN: synthetic oligonucleotides. Values are shown as mean ± S.D. from 5 determinations per substance with hemocytes from 5 different bivalves (size range 6.5–7 cm). * = significant differences to control ($p<0.05$, Student t-test).

Treatment	Control	Flagellin	PGN	LPS	PMA	Zymosan	ODN
Optical density (630 nm) per cell $\times 10^{-7}$	0.853	1.05*	1.054*	1.263	1.142*	1.686*	1.122*
± S.D. $\times 10^{-7}$	0.075	0.17	0.072	0.48	0.109	0.158	0.144

Table 2
Generation of ROS in the hemolymph of *L. elliptica* measured as formazan accumulation. LPS and Antarctic environmental bacteria from the water column (EB1: heat-killed, EB2: alive) and isolated directly from *L. elliptica* and the surrounding sediment (EB3: heat killed, EB4: alive) were used as stimulants. Values are shown as mean ± S.D. from 6 determinations using hemocytes (size range 6.5–7 cm) from 6 individual bivalves. * = significant differences to control ($p<0.05$, Student t-test).

Treatment	Optical density (630 nm) per cell $\times 10^{-7}$	$\pm$S.D. $\times 10^{-7}$
Control	0.493	0.042
LPS	0.585	0.134
EB1	0.560	0.070
EB2	0.584	0.096
EB3	0.562	0.076
EB4	0.635*	0.080

significantly higher formazan formation per cell compared to controls (Table 1). Exposure of hemocytes from large animals to LPS also stimulated the production of formazan per cell compared to non-treated controls, although non-significantly.

Exposure of hemocytes from large animals to living or heat killed natural cultivated water column and sediment bacteria, as well as LPS (positive control), induced formazan formation over control levels (Table 2), but was only significant in case of life bacteria from the surrounding sediment (EB4).

3.4. Size/age effects

Hemocyte number was significantly higher in larger compared to smaller individuals (Fig. 5) directly sampled from the field. Control levels of formazan-production per cell by non-stimulated hemocytes were higher in the smaller compared to larger bivalves and also the zymosan stimulated increase in ROS generation was higher in hemocytes from smaller than from larger sized bivalves (Fig. 6).

3.5. Antibacterial capacity

No inhibition of natural bacterial growth was observed using gill homogenates or pure hemolymph. In both assays bacterial communities, isolated from the environment of *L. elliptica* and exposed to the homogenates or hemolymph on agar plates or in suspension, showed similar colony formation as the seawater treated controls.

3.6. Whole animal experiments

Hemocyte numbers in the hemolymph of animals kept in the different laboratory treatments decreased over the time course of the experiment compared to control animals taken directly after 10 days of acclimation to laboratory conditions before beginning the treatment (Fig. 7). Larger individuals of this control group (Fig. 7B) had again higher HC than smaller individuals (Fig. 7A).

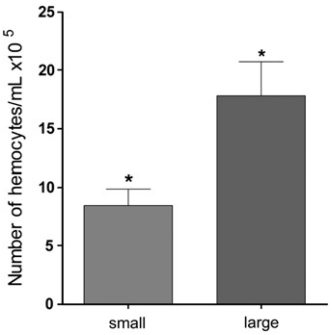

Fig. 5. Hemocyte counts (cells/ml) of small and large untreated *L. elliptica* individuals. Values are mean ± S.E.M, N = 6–7 individuals per group. * = significantly different between groups, $p<0.05$ (Student t-test).

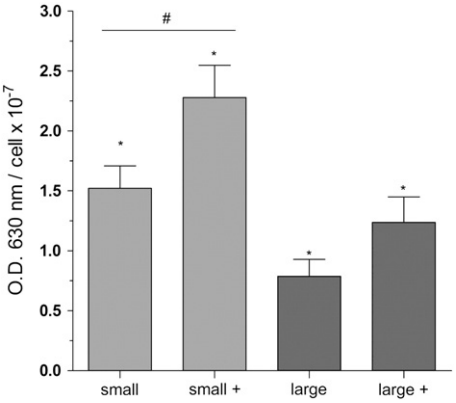

Fig. 6. Hemocyte ROS generation (formazan formation) of different *L. elliptica* size groups, untreated and in response to zymosan (+). Values are mean ± S.E.M., N = 5–6 individuals per group and treatment. * = significant difference between size groups within the respective treatment, # = significant difference between treatments, $p<0.05$ (Student t-test).

Starvation had a strong effect on HC compared to high food loads (Fig. 7). Especially in the large size group, hemocyte numbers of fed animals (Fig. 7B, group F) were significantly higher compared to starved animals after 23 and 44 days of treatment (Fig. 7B, group S). Small and large starved animals displayed higher numbers of circulating hemocytes after acute injury (2 days after the injury event, group S/I, Fig. 7A and B) compared to non-injured animals of the same alimentation treatment (Fig. 7A and B, group S). Contrarily, hemocyte counts in injured and non-injured animals of the high food load treatment were not significantly different. HC in the injured and starved animals returned to non-injury levels after three weeks of regeneration.

4. Discussion

The hemolymph of the Antarctic soft shell clam *L. elliptica* contains two major types of hemocyte cells, with less than 10% large granulocytes (type 1) and over 90% small agranulocytes (type 2). When plated on microscope slides, hemocytes attach and form complex networks. *L. elliptica* hemocytes displayed the same immune responses described in other bivalves such as phagocytosis and ROS generation. Agranulocytes were capable of phagocytosis and incorporated experimentally added yeast cells.

ROS production in response to different immune stimulants of microbial origin was measured in whole hemocytes population. Similar as shown in studies of bivalve, crab and lobster hemocytes (Anderson, 1994; Moss and Allam, 2006), a basal level of superoxide anion production happens in non-stimulated hemocytes. Baseline levels of ROS formation might reflect stress from the experimental procedure of hemocyte collection (Anderson, 1994; Moss and Allam, 2006), but also a baseline of autophagic or phagocytic activity, presumably targeted to the bacterial microflora usually present in the hemolymphatic fluid of healthy animals with an open circulatory system (Sizemore et al., 1975; Tubiash et al., 1975; Adema et al., 1991; Garnier et al., 2007). Hemocytes derived ROS formation among bivalve species is variable just as the response to different stimulants which was also the case for *L. elliptica* (Anderson, 1994; Hine, 1999; Donaghy et al., 2009). We found high differences in the baseline levels between the different experimental sets in the present study (Tables 1 and 2) so that absolute values should be taken with caution. Control and stimulation experiments were therefore always undertaken in parallel and with the same batch of hemocytes to make sure that the observed changes in ROS generation are caused by the stimulants and not due to other factors like inter-individual variations. The most vigorous response of *L. elliptica* hemocytes was against zymosan, a protein–carbohydrate complex derived from yeast cell walls, previously found to elicit a

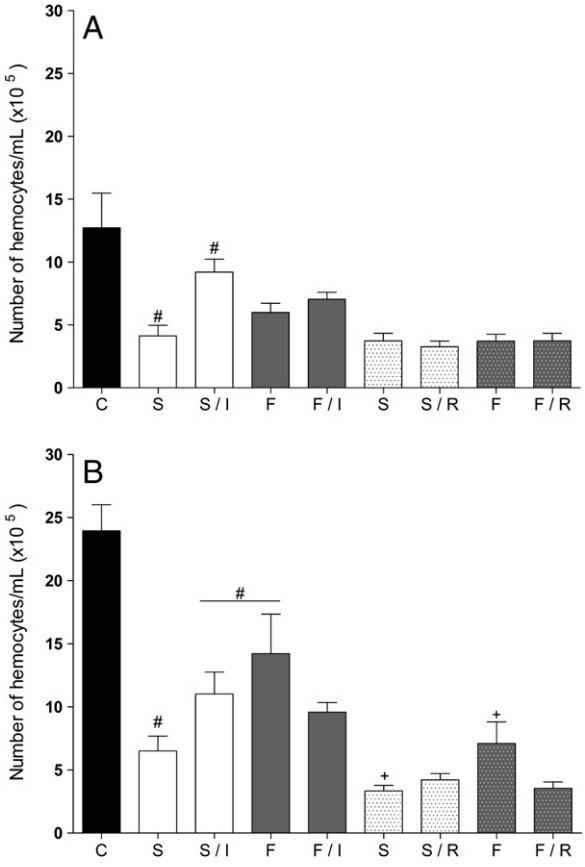

Fig. 7. Hemocyte counts (cells/ml): small (A) and large (B) *L. elliptica* individuals were sampled after 10 days of laboratory acclimation (C, black bars), 23 days under "food" (F, grey bars) and "starvation" (S, white bars) conditions and acute (2 days since infliction) injury (S/I and F/I), as well as 44 days of food (F, pattern grey bars) and starvation (S, pattern white bars) and recovering (S/R and F/R) from the previous inflicted injury. Group means ± S.E.M, N = 5–12 individuals. #, + = significant differences between groups marked with the same symbol ($p<0.05$, Student t-test).

reproducible immune response in bivalve studies (Hegaret et al., 2003; Bettencourt et al., 2007). All other tested stimulants did only trigger a minor elevation of ROS generation compared to unstimulated levels (Table 1). The differences in ROS generation in response to different stimulants in our study may indicate stimulation via direct or indirect pathways. Some microbial stimulants may depend on indirect opzonization mechanisms, whereas others, such as zymosan and LPS, are apparently recognized directly by the receptors on the hemocyte membranes. The possible link between the mechanism of foreign particle recognition and induction of phagocytosis, which may involve ROS production, still remains unclear in bivalves. Incubation of hemocytes with cultivated natural environmental bacteria resulted in only marginal ROS generation. However, microscopical observations of hemocytes incubated with the different stimuli revealed morphological changes and effects on aggregation of hemocytes even in treatments where only a marginal oxidative burst reaction was observed with the NBT assay (Fig. 4). This shows that other immune responses protect against bacterial invaders, e.g. in response to flagellin treatment. Especially in the LPS treatment a complete lysis of hemocytes was observed after 3 h, indicating a strong immune response following morphological alterations which eventually end with the complete destruction of the intoxicated hemocytes. Respiratory burst and phagocytosis represent only two parameters of the bivalve's immune repertoire. In addition hemocytes are known to secrete antimicrobial peptides into the hemolymph for bacterial recognition and killing (Pruzzo et al., 2005). In *L. elliptica* however no antimicrobial activity of the hemolymph could be observed when tested against different environmental bacteria. In general, immune stimulation of *L. elliptica* hemocytes by different stressors resulted in rather low ROS generation compared to other bivalve studies (Anderson, 1994; Bettencourt et al., 2007), and antimicrobial activity against environmental bacteria was not detectable. Intrinsic (age and size) and extrinsic (food and injury) factors seem to be important modulators of the *L. elliptica* immune response. Small clams had a lower number of hemocytes but higher basal and stimulated (zymosan) ROS generation per cell compared to larger/older clams, speaking for a decrease in the immune response capacity over lifetime.

Experimental changes of extrinsic factors such as food or injury showed that the number of hemocytes in *L. elliptica* is influenced by the animals' physical state and subject to environmental changes. The observed high numbers of hemocytes in animals 10 days after collection from the field compared to those kept for 3 and 6 weeks in the laboratory may reflect a higher need of large hemocyte numbers under natural conditions, with presumably higher environmental stimulation by variable biotic and abiotic factors, which are absent under controlled laboratory conditions. There was a tendency of lower hemocyte numbers in starved compared to fed individuals, which might be due to a reduction of energy allocation to hemocyte proliferation under starvation, or lower microbial numbers in the filtered seawater in the starved animal tanks. Two days after an experimental injury event, HCs increased in starved young and old individuals, whereas the elevation was only minor in fed small individuals, and not detected in large fed individuals. Studies investigating the effect of starvation or caloric restriction on hemocyte numbers and the immune system are still scarce and inconsistent. In the oyster *Crassostrea gigas*, Hégaret and Wikfors (2005); Hégaret et al. (2004) found either no or rather a negative influence of starvation on the immune status (e.g. lower hemocyte number, proliferation and respiratory burst) compared to well fed individuals. In case of caloric restriction a general positive effect on the immune system is anticipated throughout the animal kingdom (Nikolich-Zugich and Messaoudi, 2005). Our results of the hemocyte counts point towards a higher responsiveness to injury of animals kept under the starvation regime; the underlying mechanisms are however unclear and further studies are necessary to strengthen this observation.

In any event, hemocytes seem to be involved in wound healing and are known to be important for wound sealing to avoid loss of hemolymph and invasion of microorganisms upon injury, as well as to form a matrix for the regeneration of epithelial cells as described for *Pinctada fucata* (Acosta-Salmón and Southgate, 2006). Increased numbers of free floating hemocytes upon injury in small individuals may result from migration of previously stationary tissue cells to the hemolymph, rather than increased proliferation of circulating hemocytes, as already suggested by Coles et al. (1995). So far, no one has studied the generation of hemocyte cells, and this aspect thus remains purely hypothetical. Reduction of HC to control levels 3 weeks post injury indicates these cells to represent only a first line of defense after serious impact.

In summary, hemocytes of *L. elliptica* perform a common immune response of phagocytosis and ROS generation as well as bacterial aggregation, previously observed in bivalves. Physiological conditions such as size, age, starvation and injury modulate hemocyte numbers and hemocyte ROS generation in *L. elliptica*. In future, increased water temperatures (Whitehouse et al., 2008) and ongoing eutrophication especially in the vicinity of Antarctic field stations may produce more intensive bacterial growth and alter microbial species composition. Intensified coastal run off (Dominguez and Eraso, 2007) can further enhance microbial challenge in King George Island coastal areas and additionally the higher content of inorganic sedimentary material in the water column can alter *L. elliptica* physiology as it can starve the animals or influence filtration and respiration (Philipp et al., in press). All these factors together with the predicted increase in ice scouring frequency which can break the shell and injure *L. elliptica* (Philipp et al., in press) are bound to challenge the immune system of the

Antarctic soft shell clam and may contribute to changes in species survival and population composition under current climate change conditions in West Antarctic coastal ecosystems.

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