

**The bivalve *Laternula elliptica*:
physiological and molecular response to changing coastal
Antarctic environments**

Dissertation

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1. INTRODUCTION

1.1 Coastal benthic environments of the Western Antarctic Peninsula

The Western Antarctic Peninsula (WAP), which extends from the Bellingshausen Sea to the northern tip of the peninsula (Fig. 1) is a productive coastal ecosystem. It supports large numbers of marine mammals and birds, Antarctic krill as well as a diverse benthic macrofauna (Barnes and Clarke 2011; Ducklow et al. 2007; Clarke 2008). The benthic fauna can reach a biomass of several kg wet weight per m² and is dominated by sessile suspension-feeders such as sponges, bivalves, cnidarians, bryozoans, ascidians and echinoderms (Barnes and Peck 2008; Gili et al. 2001; Thomas et al. 2008). The marine ecosystem is characterized by the seasonal formation and melt of sea ice cover, winters with low primary productivity and productive summers with peaks of phytoplankton blooms (Barnes and Clarke 2011; Schloss et al. 2012).

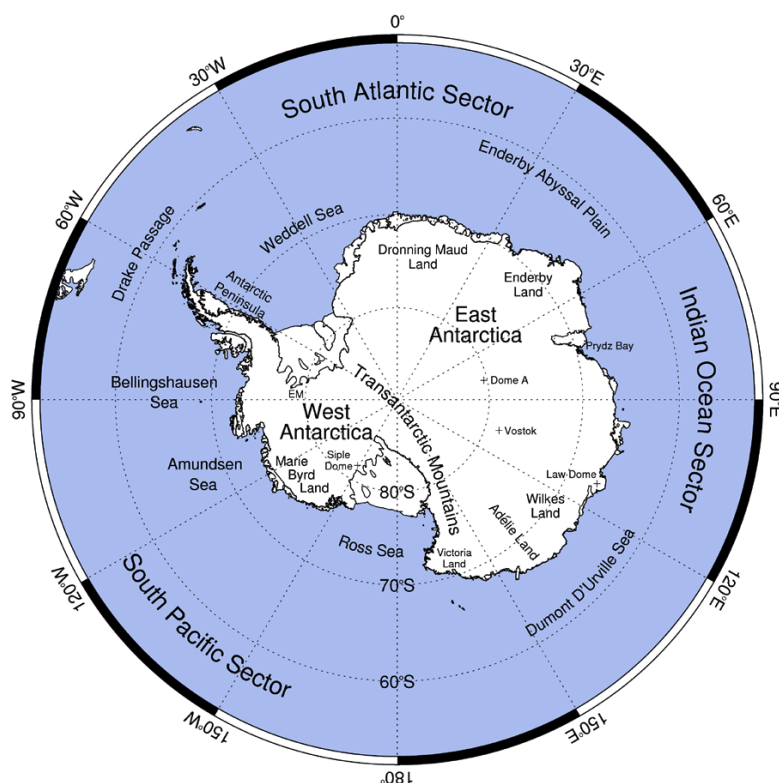


Figure 1: Map of Antarctica showing selected topographic features (Turner et al. 2009).

The Antarctic Circumpolar Current (ACC) forms a hydrological barrier and separates the Antarctic shelf areas from warmer waters and more temperate atmospheric conditions in the

north (McClintock et al. 2008; Turner et al. 2009). Due to the long reproductive isolation of the marine Antarctic community by the ACC and by distance from other shelf areas, a number of species appear to be endemic to the Antarctic and became adapted to very low and seasonally only marginally varying seawater temperatures (ranging from winter temperatures close to the freezing point at $\sim -1.9^{\circ}\text{C}$ to summer values of $\sim +1.5^{\circ}\text{C}$) (Barnes and Kaiser 2007; Barnes and Peck 2008; Schloss et al. 2012).

As a consequence, a number of polar benthic species grow slowly with long generation times, have low metabolic rates, lower activity compared with related or ecologically similar temperate species and exhibit narrow physiological temperature limits of survival. These species are proposed to have poor abilities to adapt to changing conditions by physiological adjustments or by producing new genetic adaptations. Now, as the climate at the WAP shifts in a drastic way, these species appear particularly vulnerable to rapid changes of environmental conditions (Peck 2005; Clarke et al. 2007; Thomas et al. 2008).

1.2 Climate change

1.2.1 Recent environmental changes at the WAP

The Antarctic Peninsula ecosystem is currently subject to rapid environmental changes and belongs to the fastest warming regions on earth (Turner et al. 2009; McClintock et al. 2008; Vaughan et al. 2003). Time series records of 16 Antarctic stations, mostly beginning in the 1950s, reported significant warming trends around the WAP (Fig. 2), with annual mean air temperatures locally rising by $\sim 0.6^{\circ}\text{C decade}^{-1}$ (Turner et al. 2005).

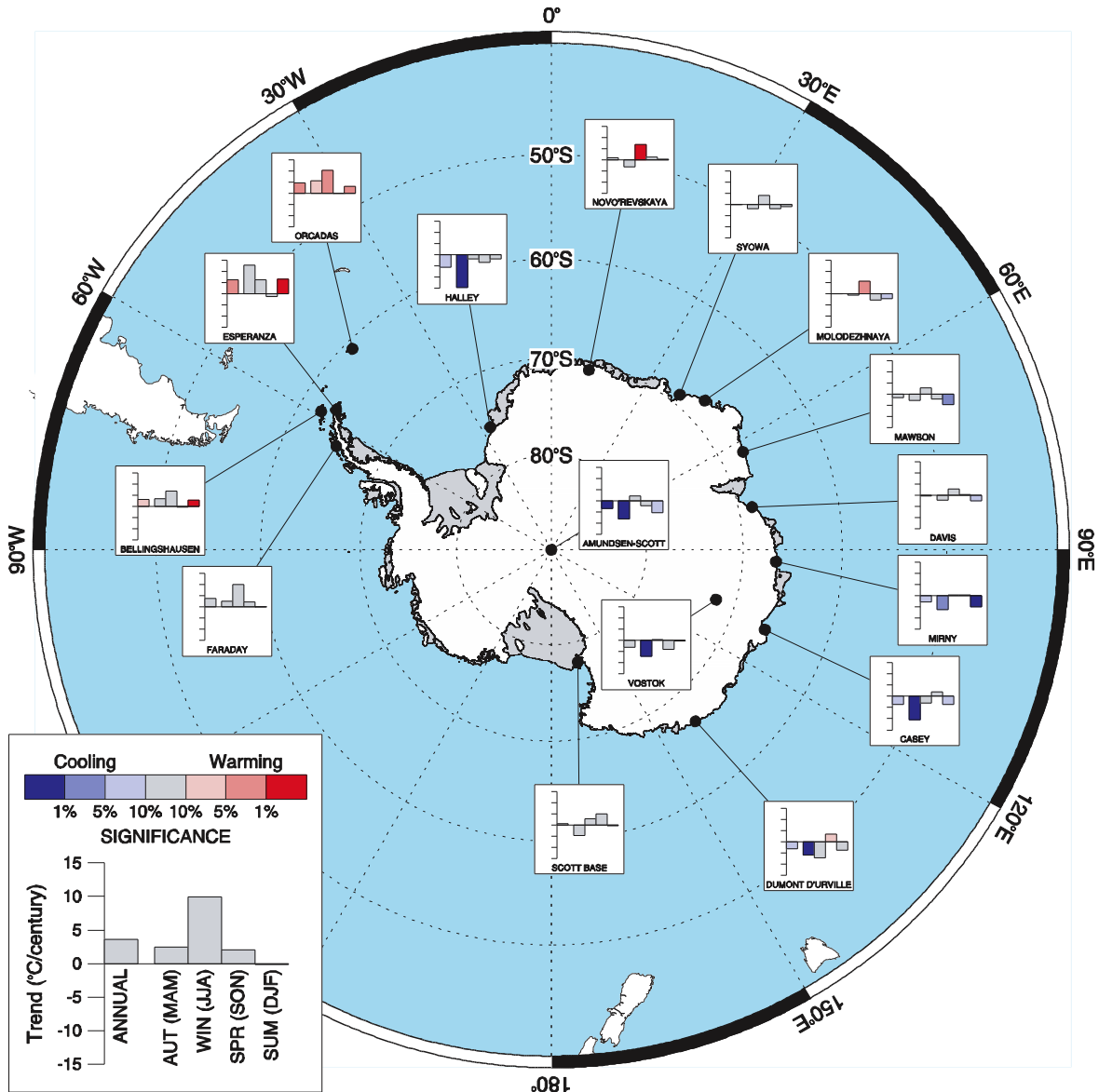


Figure 2: Antarctic near-surface temperature trends from time series data for the period 1971-2000 (Turner et al. 2005; Turner et al. 2009).

Reconstructions for the second half of the last century using land- and ship/buoy-based recordings or infrared satellite data from Antarctica confirmed the most striking warming trends of near surface air temperatures to manifest at the Antarctic Peninsula and extending to parts of West Antarctica (Fig. 3A and 3B) (Steig et al. 2009; Chapman and Walsh 2007).

Similar results are based on stable isotope recordings from ice-cores taken from the Antarctic Peninsula region and West Antarctica. Here, a mean increase in temperature of $1^{\circ}\text{C century}^{-1}$ was reconstructed for the period 1900 to 1999 (Schneider and Steig 2008), whereas Thomas et al. (2009) detected an overall local rise of $\sim 2.7^{\circ}\text{C}$ since the 1950s from a southern WAP ice-core.

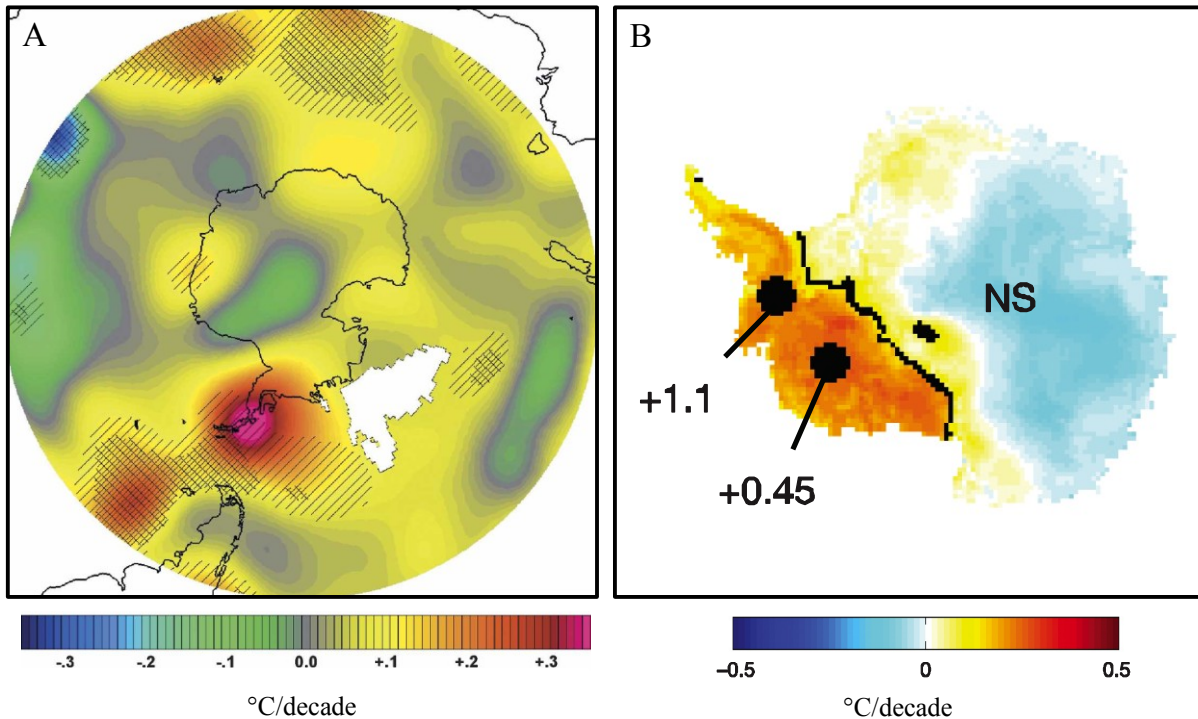


Figure 3: Spatial pattern of air temperature trends around Antarctica ($^{\circ}\text{C decade}^{-1}$). **A:** linear trends of annual mean surface air temperature (period 1958-2002), gridded from data of land surface stations, automatic weather stations and ship/buoy measurements. Significant trends are indicated by hatching (statistical significance threshold $>95\%$ single hatching, $>99\%$ crosshatching) (Chapman and Walsh 2007). **B:** reconstructed for the period 1969-2000, using infrared (T_{IR}) satellite data (Steig et al. 2009).

The atmospheric warming trends appear to have a great influence on the WAP coastal environment. Changes become evident in surface and near surface water temperatures that have warmed by more than 1°C in summer over the past decades (Whitehouse et al. 2008; Schloss et al. 2012; Meredith and King 2005).

It has been suggested, that rising surface water temperatures at the WAP are not only linked to the continued local atmospheric warming but also to oceanographic changes such as increased upwelling or warming of Circumpolar Deep Water (CDW) of the ACC which is oceanic source for coastal waters of the WAP; or warming of upper layers of the ACC (Clarke et al. 2007; Ducklow et al. 2007; Böning et al. 2008; Clarke et al. 2009).

On land, these atmospheric and oceanographic trends have a strong impact on the erosion and moving dynamics of glaciers and ice shelves (Fig. 4) (Turner et al. 2009; Pritchard et al. 2012). Large parts of the marine WAP experience local air temperatures above freezing point in summer and periodically during spring and autumn. Therefore these parts experience frequent melting periods, which strongly affect the mass balance of the ice sheets. For the second half of the twentieth century, long term records from the WAP show increasing annual

time spans of these summer melting seasons, with rising numbers of positive degree-days (PDD) of up to 74% at Faraday/Vernadsky Station between 1950 and 2000 and similar rates were found for Rothera or Bellingshausen (Vaughan 2006). As a consequence, there was a transition from a mean advance (62% of all investigated glaciers were advancing from 1945 – 1954) to mean retreat of glaciers (75% of all investigated glaciers were retreating from 2000–2004). During the past decades, the retreat rate of 212 of 244 observed marine tidewater glaciers and ice shelves (87%) around the WAP accelerated (on average since 1953) (Cook et al. 2005; Turner et al. 2009).

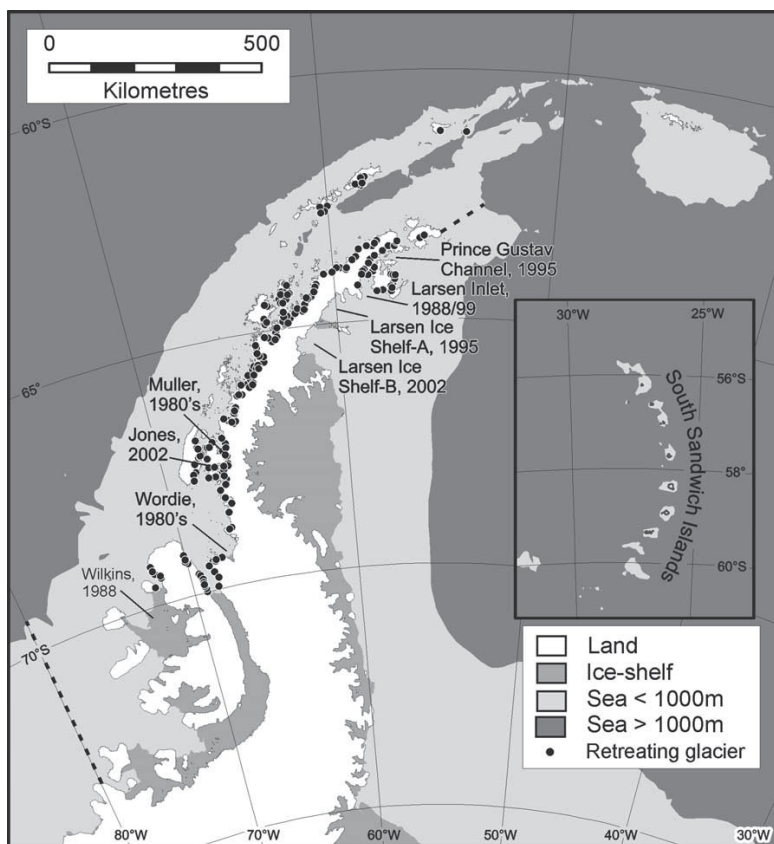


Figure 4: Map of the Antarctic Peninsula region. Shown are glaciers that have retreated in the last half century and ice shelves that have collapsed, or significantly retreated, in the last 20 yr (Smale and Barnes 2008).

Calving of maritime glaciers and ice shelves generates large amounts of ice masses. These are ranging from ice blocks to icebergs, which are floating around the marine Antarctic and can strongly affect the sea beds and coastlines (Gutt 2001; Barnes and Conlan 2007). Further, the present increase in air temperature and further predicted changes in precipitation pattern around the WAP lead to changing characteristics and absolute increases of glacial runoff (Turner et al. 2009; Vaughan 2006; Pritchard and Vaughan 2007). In nearshore environments, the freshwater runoff from glacier melting has an impact on water column stability, as it is

causing freshening and can increase stratification (Schloss et al. 2002). Increasing run off of suspended fine grained matter in front of a melting glacier leads to the formation of runoff plumes of rock flour and regions of increasing turbidity and sedimentation rates (Schloss et al. 2012; Barnes and Kaiser 2007; Hass et al. 2010).

The ongoing environmental changes at the WAP already affect the ecosystem at various levels. First impacts of climate change are indicated by local alterations in biomass and species composition at the base of the food chain such as planktonic primary producers or zooplankton like krill *Euphausia superba* (Montes-Hugo et al. 2009; Clarke et al. 2007; McClintock et al. 2008)

1.2.2 Specific environmental changes around the study site: King George Island/Isla 25 de Mayo

King George Island/Isla 25 de Mayo (KGI) lies at the northern tip of the WAP and is the largest of the South Shetland Islands (Fig. 5). Here, annual mean atmospheric temperature has risen on average 1.1°C over 49 years (period 1947 to 1995) (Ferron et al. 2004). In Potter Cove, a shallow fjord-like environment in the southwestern KGI (Fig. 5), mean annual air temperatures increased by 0.66°C during 1991 and 2009. Within the same period, the mean annual sea water temperatures warmed by 0.32°C per decade (Schloss et al. 2012).

Most areas of King George Island are covered by glaciers. Measurements over a short period from 2002 to 2006 showed a significant increase in specific discharge volume [$\text{hm}^3 \times \text{km}^{-2}$ glacier catchment area] at a confluence point of 5 glacial springs close to the Uruguayan Station Artigas (south-western King George Island) (Dominguez and Eraso 2007). Here, almost a doubling of the annual time period of discharging waves was registered, but further time series are needed to approximate a long term trend of warming (Dominguez and Eraso 2007; Bers et al. 2012).

From 2001 onwards, Dominguez and Eraso (2007) observed the appearance of new moulins and cracks as well as an increase in the speed of glacier sliding and erosion. Based on a recent study on glacier and mass balance changes, using satellite data, the lost glaciated area comprised of about 20 km² during years 2000 - 2008, corresponding to ~1.6% of the whole ice-covered area of the island. Also the large tidewater glacier Fourcade Glacier, which is enclosing large parts of the Potter Cove by ice cliffs, is drastically disintegrating and retreating (Fig. 5) (Braun et al. 2001; Rückamp et al. 2011). From 1956 – 1989, the ice shield retreated by a mean of ~ 400m and an area of 360.000 m² (Park et al. 1998). Additionally, during a period of 11 years (1997/97 – 2008/09), consistent surface lowering of the

independent ice cap Bellingshausen Dome (Fig. 5) was observed at elevations below 270 m above WGS84 ellipsoid (Rückamp et al. 2011). The reasons for the drastic changes are not completely understood, however, due to the characteristics of the retreat, changing climatic conditions are the most probable trigger (Park et al. 1998; Bers et al. 2012; Braun et al. 2001).

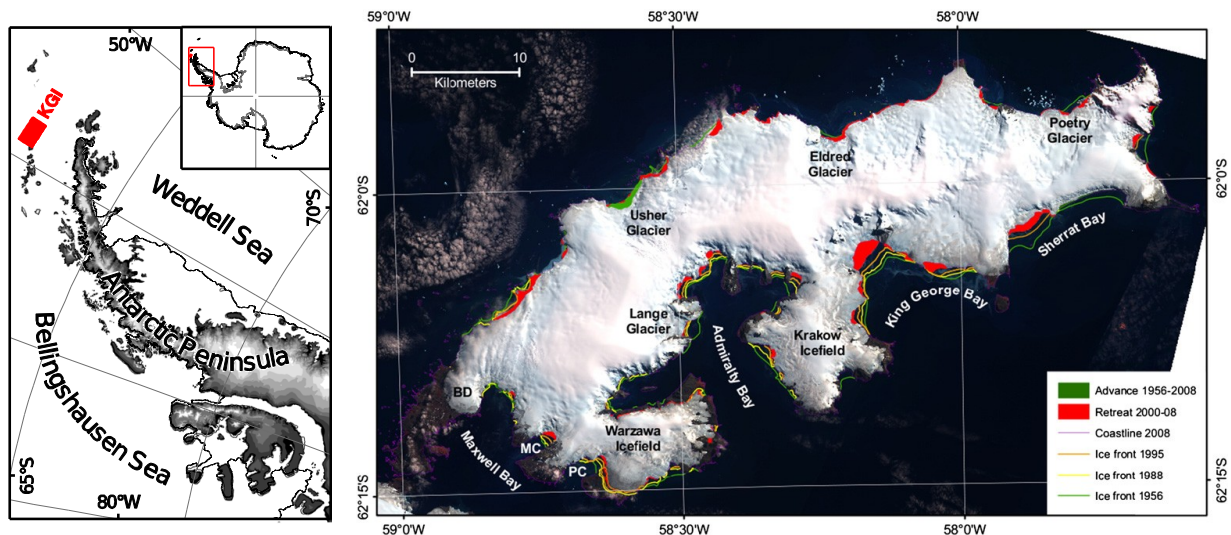


Figure 5: Glacier retreat on King George Island (KGI) since 1956 (top image). BD: Bellingshausen Dome, PC: Potter Cove and MC: Marian Cove. Together with left image taken from Rückamp et al. (2011).

In Potter Cove, the strong input of turbid melt water has a local impact on total suspended particulate matter (TSPM), salinity and water column stratification. In the inner cove, a significant long-term trend of increasing summer TSPM concentrations during 1991 and 2009 was detected in shallow waters (0 to 10m) (Schloss et al. 1997; Schloss et al. 2012; Schloss et al. 2002). In the adjacent Maxwell Bay (King George Island), mass accumulation rates of sediments almost tripled since the 1940s ($0.66 \text{ g cm}^{-2} \text{ year}^{-1}$ in 2006). Changes were reported from a sediment core record and linked to the rapid glacier retreat (Monien et al. 2011).

In Potter Cove, glacial discharge of mineral suspension and ice scouring are thus major factors potentially affecting the nearshore benthic community (Sahade et al. 1998; Torre et al. 2012). A chronic physical disturbance by both stressors is anticipated to particularly influence physiological functions and population structure of sessile and filter feeding species such as the Antarctic soft shell clam *Laternula elliptica*, which was investigated during the present study.

1.2.3 Nearshore impact of environmental changes: ice scouring

For coastal benthic communities of the Antarctic, a major force of catastrophic disturbance is through the mechanical impact of ice. In particular, icebergs and ice blocks, drifting around and scouring the seabed and shorelines can result in frequent and serious damage to hundreds of square meters of the benthic habitats down to 500 m water depth (Fig. 6 and 11).

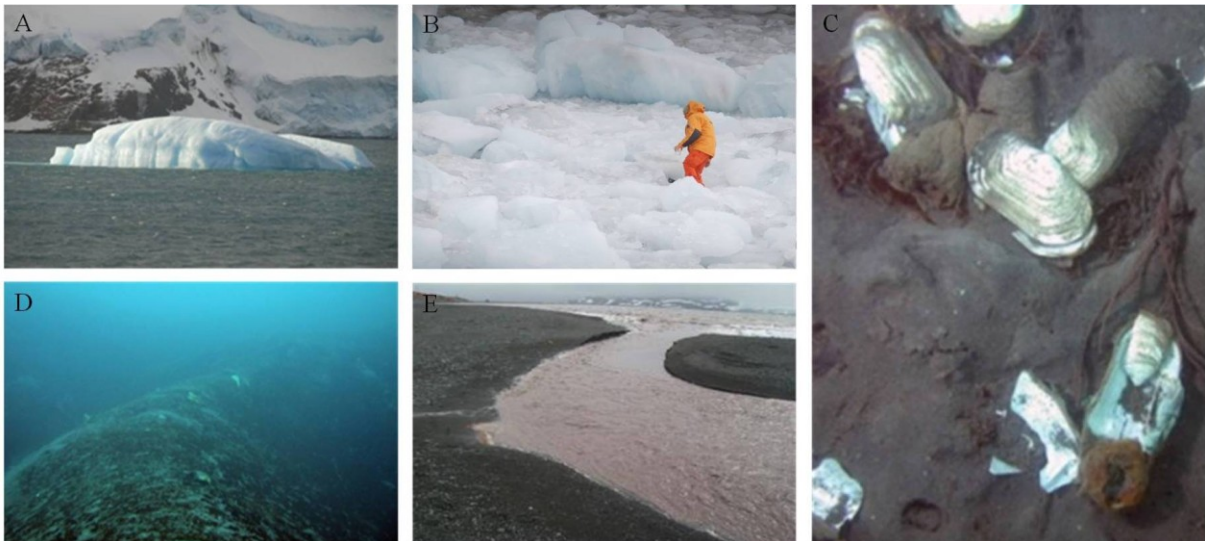


Figure 6: Ice scouring and glacial runoff at Carlini Station, King George Island, WAP. **A, B:** drifting and grounding iceberg and ice blocks, **C:** *Laternula elliptica* individuals removed from sediments and injured by ice scouring, **D:** iceberg scouring mark and shells of *L. elliptica* being removed from sediments, **E:** runoff stream originating from the local Fourcade glacier (pictures taken by E. Philipp, D. Abele and G. Husmann).

Generally, little is known about the dynamics of ice scour damage around the Western Antarctic Peninsula (WAP) region. The generation of free floating ice masses by ocean-connected ice fields, however, is likely to further increase due to ongoing ice shield disintegration. In addition, the collapse of glacier fronts or complete ice shelves is triggering an accelerated flow of subsequent glacier masses (Turner et al. 2009; Thomas et al. 2004; Pritchard and Vaughan 2007). Simultaneously the duration and extent of the WAP winter sea-ice is declining. The latter is negatively correlated with the frequency of ice scouring by preventing floating icebergs from moving (Abram et al. 2010; Stammerjohn et al. 2008). Therefore, the WAP benthic ecosystems are potentially affected by a near future scenario of both more frequent and extended annual ice scouring (see also Fig. 7) (Barnes and Conlan 2007; Barnes and Souster 2011; Smale et al. 2008b).

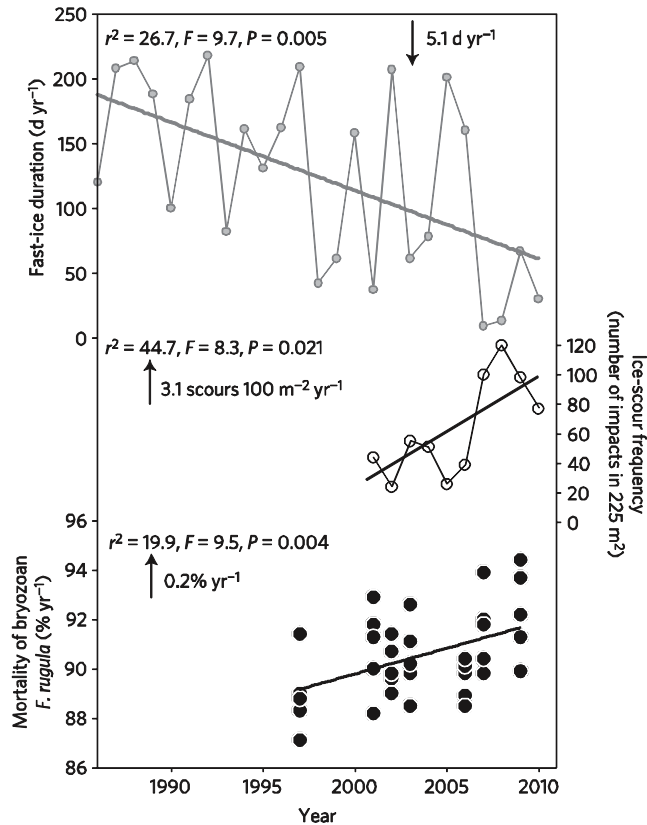


Figure 7: Impact of ice scouring on a sessile species of the Antarctic benthos (bryozoans *Fenestrulina rugula*) at Rothera station, WAP: Duration of fast-ice (frozen sea surface), ice scour rates and mortality. Shown are significant regressions (Barnes and Souster 2011).

Data sets concerning ice scouring frequencies are still scarce and interpretation is difficult due to inter-annual variation. The longest published record of iceberg scouring to the benthos at present was derived near Rothera station (Fig. 7). Here a significant increase of scouring events by grounding icebergs ($3.1 \text{ scours } 100\text{m}^{-2}\text{yr}^{-1}$) during the past decade was observed (Barnes and Souster 2011). The frequency of ice damage to the benthos is, however, likely to be much higher in areas nearby tidewater glaciers calving into shallow coastal WAP areas.

Scouring ice masses have a significant impact on the coastal benthic communities of the Antarctic, as they imply physical injury and high mortality (Barnes and Souster 2011; Gutt 2001; Barnes and Conlan 2007) (Fig. 7). Due to frequent ice scouring disturbances, populations can be completely destroyed or held at a permanent state of re-organisation (Peck et al. 1999). After sea bed impact due to a grounding iceberg Peck et al. (1999) reported a nearly complete removal of the meio- and macrofauna from the seabed (90 and 99.5% respectively). Damage by grounding ice can lead to a marked temporal and spatial zonation of benthic communities (Almeida et al. 2005; Barnes 1999; Barnes and Conlan 2007). This zonation effect and the linked changes in biodiversity and abundance of benthic flora and

fauna can be in particular strong close to calving glacier fronts (Hyland et al. 1994; Wlodarska-Kowalczyk and Pearson 2004).

Grounding ice can unearth and remove infauna species such as *L. elliptica* from their sedimentary environment. Motile infaunal species appear to be better adapted to survive impacts by ice scouring, as mobility or the ability to re-burrow appears to be of substantial importance to resettle and avoid predation. Hence, a strong impact of ice scouring on less mobile or sessile and slow growing species such as large bivalves was observed (Barnes and Peck 2008; Hinchey et al. 2006; Peck 2005). On the physiological level, frequent ice disturbances potentially shift the individuals' energy allocation from processes such as growth or reproduction to survival and maintenance of basic functions such as ice scouring avoidance by active reburial, prolonged periods of siphon retraction, shell- and tissue-repair or immune response due to injury.

It is not clear, however, to what extent the above mentioned consequences of frequent ice disturbance may increase physiological stress levels in *L. elliptica*. Moreover, little is known about how individual animals of the polar nearshore benthos might react physiologically to frequent ice disturbance, and whether it leads to shifts in the populations' structure.

1.2.4 Nearshore impact of environmental changes: sediment discharge

During periods of glacial discharge, melt water creeks transport increased amounts of inorganic sediments into nearshore coastal environments of the WAP (Fig. 6E, see also 1.2.2 for Potter Cove/King George Island). Hence sedimentation rates increase and light penetration of the water column is affected (Barnes and Kaiser 2007; Hass et al. 2010; Schloss and Ferreyra 2002). If the present warming continues, one can expect increased discharge of turbid meltwaters and further increasing sedimentation rates affecting the nearshore coastal environments (Hass et al. 2010; Smale and Barnes 2008). Long time exposure to inorganic turbidity can influence physiological functions particular in filter feeding organisms and cause higher mortality rates and finally set distribution limits (Aldridge et al. 1987b; Ellis et al. 2002; Stevens 1987; Torre et al. 2012). High sedimentation rates further result in the formation of unconsolidated sediments and thus in an unstable habitat. This effect can be further enhanced by additional sediment destabilisation and resuspension of sediments in the water column (Fig. 12) close to a calving glacier and by ice scouring (Gilbert et al. 2002; Turner et al. 2009). Frequent resuspension, high deposition rates and sedimentary movements can lead to partial coverage of animals (Fig. 12), potentially cause stress conditions such as

hypoxia; or can hinder the larval settlement (Norkko et al. 2002; Thrush et al. 2003; Wlodarska-Kowalczyk et al. 2005; Philipp and Abele 2010).

Increased turbidity can lead to negative energetic consequences by affecting physiological parameters such as filtration rates, as shown in bivalves (Ellis et al. 2002; Safi et al. 2007; Madon et al. 1998). Suspended inorganic particles potentially impair filter feeding processes by a reduction of ciliary velocity or damage of gill filaments (Stevens, 1987; Shin et al., 2002). Studying food uptake in the bivalves *Atrina zelandica*, *Dreissena polymorpha* or *Spisula solidissima*, the efficiency of the animals to select and assimilate utilizable food particles was reduced, which in the first line was a consequence of the uptake of enlarged fractions of low nutritional value. Therefore the amount of digestible food which is actually taken up via filter feeding can decrease under high inorganic turbidity, as ingested plankton or organic particles are “diluted” with an increased amount of inorganic particles (Robinson et al. 1984; Madon et al. 1998; Safi et al. 2007). Affected filtration rates and the need for increased investment in rejection of non-food particles might lead to adverse effects on the bivalves` energy allocation (Madon et al. 1998; Robinson et al. 1984; Navarro et al. 1992). Further altered quantities of the available food potentially enhance this effect, as a deep vertical dispersal of particles within the water column inhibits the penetration of light and can affect the nearshore primary production, the zooplankton and perhaps also the benthic primary production (Schloss and Ferreyra 2002; Montes-Hugo et al. 2009; Schloss et al. 2002). As these are major food sources of the benthic community, the overall energy flux to the benthos and seasonal food compositions of the animals might alter in coastal areas of frequent and strong sedimentary runoff.

Altogether, increasing periods and intensities of terrigenous runoff, linked to an unstable habitat with high inorganic turbidity and scarce planktonic food supply, are suggested to possibly lead to deteriorating feeding conditions, decreasing rates of energy acquisition and increasing costs of physiological maintenance in filter feeders such as *L. elliptica*. This as a consequence can influence the energy balance of individual animals and finally affect growth, reproduction and survival in animals affected by long-term high sedimentation.

At King George Island another consequence potentially connected with ice sheet melting and the massive runoff of eroded soil is an increased uptake of metals by filtering animals. Marine bivalves can accumulate various metals several orders of magnitude above the environmental level. These metals are dissolved in the water column and sediment pore water, mineral-bound, adsorbed to suspended particles or build a complex together with organic food components (plankton, detritus) which all can be taken up and then incorporated into the

tissues and shells of the bivalves. The latter is depending on the bioavailability of the respective element. The knowledge about bioaccumulation-pathways and the understanding of metal bioavailability in marine invertebrates is limited. However, bivalves such as *L. elliptica* are predestined for the uptake and high bioaccumulation of metals from the environment due to their filter feeding habit and permanent exposure to sediments (John and Leventhal 1995; deMoreno et al. 1997; Langston et al. 1998).

Different studies reported high environmental concentrations of metals including Al, Cu, Fe, Zn and Mn in the region of King George Island (WAP) compared to different localities of the WAP (Curville Island, Horse Shoe Island), or central Antarctica (Peter Ioy Island, Powell Island) (Alam and Sadiq 1993; Ahn et al. 1996; Abele et al. 2008a). The higher trace metal levels in sediments of this area were attributed to the local characteristics of coastal runoff fed from volcanic rock ablation as also reported for Deception Island (Antarctica), where a higher metal concentration was attributed to an active submarine volcano (Deheyn et al. 2005). This might be of particular interest, as previously detected concentrations of various metals in *L. elliptica* differ significantly among sampling sites around Antarctica, indicating that metal concentrations in this species are highly depending on the environmental setting (Ahn et al. 1996; Lohan et al. 2001). The massive runoff of eroded soil and melt water around King George Island (1.2.2) may lead to increased and chronic environmental metal exposure of local *L. elliptica* populations. A consequence of long-term exposure might thus be an increased metal incorporation into the bivalves' tissues.

1.3 Laternula elliptica, a key species of the Antarctic benthos

The Antarctic soft shell clam *Laternula elliptica* (Anomalodesmata, Laternulidae; King and Broderip, 1831) is a large (>10 cm shell length) and long-lived (>36 years) bivalve of the macro-benthos. The geographically circumpolar distributed species is endemic to the Antarctic (Philipp et al. 2008a; Brey et al. 2011; Urban and Mercuri 1998).

Around the Western Antarctic Peninsula and Antarctic islands, it locally predominates the infauna of shallow nearshore habitats. Here, populations of the sessile animals can reach total ecological biomasses of 9 kg wet weight m⁻² (Ahn 1994). In Potter Cove, the animals occur in dense patches of up to 346 individuals m⁻² (Fig. 8) (Mercuri et al. 1998; Philipp et al. 2011). By deeply burrowing into the soft sediments (frequently >50 cm), exposing only the retractable siphon-openings on the sediment surface for suspension feeding (Fig. 8), infaunal species avoid exposure to the physically harsh environment and to predators (Hardy 1972; Peck et al. 2004a; Ahn et al. 2001). Once removed from the sediments, *L. elliptica* is

consumed by predators of the epifauna, such as starfishes *Odontaster validus*, *Cryptasterias turqueti* or *Ophiurus victoriae*, nemertean worms *Parborlasia corrugates*, or gastropods *Neobuccinum* spp. (Zamorano et al. 1986; Urban and Mercuri 1998).



Figure 8: *Laternula elliptica*. Left side: individuals of different ages, removed from the sediments, right side: individuals, extending only the siphon-tips out of the sediments for feeding and respiration; inhalant and exhalant siphon-openings are visible.

L. elliptica is a hermaphrodite which spawns primarily during Antarctic summers but was also observed to spawn during autumn and winter (Urban and Mercuri 1998; Ahn et al. 2000; Powell et al. 2001; Kang et al. 2009; Bigatti et al. 2001). There is evidence for encapsulated and lecithotrophic larvae which, after external fertilization, temporarily remain in the water column to ensure widespread dispersal for maintenance of a large population (Ansell and Harvey 1997; Pearse et al. 1991; Powell et al. 2001).

In *L. elliptica* gonad development rapidly increases with shell length and age, and older individuals around 80 mm shell length appear to have the highest reproductive output (Kang et al. 2003; Urban and Mercuri 1998).

L. elliptica appears to be a key species of the Antarctic marine food chain, as it enhances the flux of organic carbon from the pelagial to the benthos. This flux is resulting from the animals' active filtration of plankton and organic matter from the water column. Populations excrete non-digested and bioavailable carbon- and nitrogen-sources (faeces and pseudofaeces) to the sea bed (Ahn 1993; Momo et al. 2002). This process likely is crucial for biodeposition

and nourishment of benthic decomposers or deposit-feeders (Norkko et al. 2001; Grenz et al. 1990; Frankenberg and Smith 1967) and might be of particular importance in plankton restricted nearshore areas and areas of periodic extremely restricted marine primary production such as Antarctica (Ahn 1993; Momo et al. 2002).

The recently elevating environmental stress levels potentially act to disturb physiological processes and finally physiological homeostasis in *L. elliptica*. This might finally lead to impaired population fitness. The majority of studies about environmental impact on *L. elliptica* focused on consequences of increasing water temperatures. Antarctic marine ectotherms such as *L. elliptica* are adapted to low temperatures and narrow windows of thermal variation and the general warming trend or peaks of rising temperatures will likely affect the animals' physiology at different organismic levels (Barnes and Peck 2008; Peck 2005; Peck et al. 2010). Indeed, metabolic rates such as oxygen consumption or heart beat were found to increase in *L. elliptica* under experimental warming. The species lost the ability to perform essential activities (such as burrowing) when experimentally treated by temperatures of 2-3°C (Morley et al. 2009; Peck et al. 2007; Peck et al. 2004b). At 2.2°C, Morley et al. (2009) reported a 50% loss of the animals' ability to reburrow, which is close to current environmental summer maxima (Barnes et al. 2006; Schloss et al. 2012). The loss of physiological performance was followed by increased mortality rates at 4-5°C. By decreasing oxygen contents Peck et al. (2007) further experimentally reduced the temperature limit of burial by ~ 2°C. This indicates how sensitive the animals might react to rising and cross acting stressors.

In particular sessile filter feeders of the shallows around the WAP, such as *L. elliptica*, are in first line to experience additional change such as increasing glacial runoff and more frequent impact by floating ice. Hence, the focus of this work was directed towards environmental stress resulting from high sediment runoff and effects of ice scouring.

1.4 Oxidative stress responses in bivalves

In marine invertebrates, physiological stress induced by most forms of severe external impact can be accompanied by oxidative stress. The latter is caused by an intracellular imbalance between generation and physiological compensation of reactive oxygen species (ROS) such as hydrogen peroxides, singlet oxygen or hydroxyl radicals. During normal aerobic metabolism ROS are generated by mitochondria and the endoplasmatic reticulum, and balanced by the antioxidant defense system. Under phases of oxidative stress, the production

of ROS overwhelms the antioxidant defense and can cause damage of macromolecules like lipids, proteins or nucleic acids (Lesser 2006; Stadtman 1992).

As a consequence of such stress related ROS damage, specific products such as protein carbonyls, DNA adducts or lipofuscin are formed. If not repaired or removed by cellular processes such as autophagy or diluted by cellular proliferation, these waste products of oxidative damage can accumulate and interfere with cellular functioning (Terman and Brunk 2004; Brunk and Terman 2002; Catalgol et al. 2012).

As an example, an increased uptake and cellular accumulation of potentially reactive substances such as metals from the environment is believed to induce oxidative stress in marine animals (González et al. 2012; Regoli 2012). Elements like Co, Cu, Fe, Mn or Zn are essential elements for cellular functioning, but if present in excess and not bound to functional proteins (such as enzymes) or metal storage proteins, metals can cause oxidative stress and other toxic effects (Viarengo, 1985; Goyer, 1997; Company et al., 2008). To what extent the above mentioned consequences of frequent physiological stress due to grounding ice or acute peaks of turbidity may further contribute to increase levels of oxidative stress in *L. elliptica*, however, is not clear. Iceberg unearthing might lead to energetic deficits and injury. Sedimentation might lead to hypoxia through clogging of respiratory epithelia.

In bivalves, the increased generation of ROS induced by extrinsic stress has two sides. On the one hand, imbalances between ROS generation and antioxidant defense can lead to oxidative stress; on the other hand, ROS production appears to be a key mechanism of the cellular immune response in invertebrates. The cellular repertoire of immunity and stress response in bivalves is still poorly understood (for review see Tomanek (2011), Venier (2011) and Philipp (2012b)). To maintain integrity, bivalves rely exclusively on an innate immune system (Bachere et al. 1995; Canesi et al. 2002). Under injury and/or infection, invading microorganisms and non-self particles are recognized and removed by humoral and cellular mechanisms. The humoral response includes biosynthesis of defense factors such as antimicrobial peptides, lysosomal enzymes or agglutinins (lectins) (Bettencourt et al. 2007; Philipp et al. 2012b). Cellular reactions such as phagocytosis or encapsulation are mediated by the hemocytes (Pruzzo et al. 2005; Donaghy et al. 2009b). These migratory cells are major actors of the invertebrates' immunity and apparently have key functions in inflammatory response, wound repair, shell formation, transport, digestion or secretion of humoral factors (Canesi et al. 2002; Donaghy et al. 2009b; Kadar 2008). A key feature of the hemocyte response consists in the massive generation of ROS ("respiratory burst"), triggered upon recognition and phagocytosis of non-self particles or bacteria. ROS generation in hemocytes

might be catalyzed by a family of membrane bound NADPH and dual oxidases (NOX, DUOX) (Philipp et al. 2012b; Saphörster in prep.). Initially, molecular oxygen is reduced to superoxide anion ($O_2^{\bullet-}$) which generates various highly reactive and potentially cytotoxic oxygen metabolites (such as hydrogen peroxides, singlet oxygen or hydroxyl radicals) (Lambeth 2004). Respiratory burst like reactions have been recorded in several bivalves (Anderson et al. 1992; Boyd and Burnett 1999; Donaghy et al. 2009a) and can also involve the generation of various reactive nitrogen species (RNS) (Ottaviani et al. 1993; Arumugam et al. 2000; Gourdon et al. 2001). The production of RNS starts with initial generation of nitric oxide radical (NO^{\bullet}) and is catalyzed by the NO synthase family (NOS) (Bogdan 2001). Little is known regarding the link between ROS/RNS generation and immune functioning, however, it was not only ascribed to phagocytosis and antimicrobial activity but also to putative functions in cell signaling (Arumugam et al. 2000; Reeves et al. 2003; Bogdan 2001; Pourova et al. 2010).

Effects of environmental impact on the cellular immunity of bivalves have been largely investigated in terms of hemocyte abundance, phagocytic activity and respiratory burst response (for review see Donaghy et al. 2009b; Auffret 2005; Canesi et al. 2002). In this context, general ecological factors such as food (Butt et al. 2007; Hegaret et al. 2004), mechanical disturbance (Ballarin et al. 2003), high temperature (Parry and Pipe 2004; Hawley and Altizer 2011) or inorganic pollution/xenobiotics (Auffret 2005; Oliver et al. 2001) were shown to critically affect the capacity for stress reactions in bivalves.

A novel aspect of bivalve physiology linking physiological stress and immune responses is the regulation of molecular effectors such as functional peptides and proteins in hemocytes and soft body tissues (Koutsogiannaki and Kaloyianni 2010; Tomanek 2011). Proteomic/transcriptomic studies in bivalves point towards a commonly induced set of stress-proteins (Tomanek 2011). These comprise heat shock proteins (HSPs) involved in the stabilisation of proteins (Clark et al. 2008; Santoro 2000), as well as molecules participating in oxidative stress regulation (Canesi et al. 2010; Monari et al. 2008; Park et al. 2009), tissue damage repair (De Decker and Saulnier 2011; Montagnani et al. 2001), tissue development (Badariotti et al. 2006; Badariotti et al. 2007; Tirape et al. 2007) or antimicrobial defence (Xu et al. 2010). However, to what extent physiological stress due to changing environmental conditions may also contribute to modulated *L. elliptica* stress reactions, is not clear.

1.5 Understanding the population response to environmental stress/Age-related sensitivities to environmental impact

Given that *L. elliptica* is a dominating component of the nearshore benthic-pelagic energy flux, an impact on the population's fitness and survival would cause cascading effects on local benthic communities inhabiting affected areas. Accumulating effects of multiple stressors such as rising temperatures, sedimentation or ice scouring potentially lead to physiological trade-offs between systemic and cellular parameters such as cellular maintenance, stress and/or immune response, reproduction, growth or regeneration from injury. In this context, knowledge about the bivalve's physiological performance under stress is required.

To investigate effects of a specific environmental disturbance on a population, it is necessary to study different age classes. For bivalves it was shown that individuals of different ages can differ in their physiological capacity to deal with stress (Hole et al. 1993; Canesi and Viarengo 1997; Philipp et al. 2008b; Schmidt et al. 2008). Age-related shifts in stress responses go hand in hand with shifts in cellular and organismic function and manifest on different levels of organization. These range from cellular alterations up to shifts in the whole animal's physiological performance.

As an example young queen scallops *Aequipecten opercularis* confronted with predators were able to close their shells more often and for longer periods than older individuals, reflecting higher aerobic or anaerobic energetic capacities in younger individuals (Philipp et al. 2008b; Schmidt et al. 2008). Within 14 experimentally investigated species of Antarctic marine invertebrates Peck et al. (2009) showed smaller/younger individuals to better survive higher temperatures compared to larger/older ones.

On a cellular level, effects of ageing in bivalves can comprise a decline of metabolic and antioxidant capacities, declining DNA repair capacity, shifts in molecular chaperone expression (HSPs) as well as increasing accumulation of oxidatively damaged macromolecules (Abele et al. 2009; Philipp and Abele 2010; Bodnar 2009). This indicates that, similar to vertebrates, older marine invertebrates might be systemically more susceptible to environmental stress. A broad scale of external stressors thus seems capable of influencing the physiological state and population structure in bivalves and also in *L. elliptica*.

However, when comparing bivalve species, the nature of ageing processes appears very different. The species ecotype appears to influence this process. Physiological ageing appears to be extremely slowed down in burrowing ocean quahogs *Arctica islandica* after sexual

maturation at ages of ~ 30 years. This might contribute to the fact that this rather inactive species can reach extraordinary life spans of 375 years and more (Abele et al. 2008b; Philipp and Abele 2010). In contrast, faster physiological aging was reported of epibenthic scallops *Aequipecten opercularis* which are actively swimming and do not live longer than 8 to 10 years (Philipp et al. 2006).

L. elliptica can reach individual ages of 36 years and more (Philipp et al. 2008a), which was attributed to a lifelong reduced mitochondrial production of reactive oxygen species (ROS) and lower levels of mitochondrial ROS production compared to a morphologically comparable species from temperate water (*Mya arenaria*), which only reaches ages of approximately 13 years (Philipp et al. 2005b; Philipp et al. 2012c).

It is not clear, if this goes hand in hand with the ability to also maintain a physiological stress-compensation response which is stable over the whole lifetime of *L. elliptica*. Peck et al. (1999) reported a higher susceptibility of older/larger *L. elliptica* to experimentally increased temperatures compared to smaller/younger individuals. This might indicate a broad decline of cellular and physiological capacities (such as metabolism) in ageing *L. elliptica*, which may lead to a declining ability to physiologically react to environmental stress. This is particularly of importance for the slow growing, long-lived and late reproductively active *L. elliptica*, as long life spans and long generation times might decelerate a genetic adaptation of the species. To what extent this is true for *L. elliptica*, and whether the ability to adapt to complex stress levels differs between ages has to be investigated.

1.6 Research aims/Outline of the thesis

The main objective of this thesis was to increase the knowledge about the physiological performance of *L. elliptica* under environmental disturbance by integrating observations from various aspects of the bivalves' biological organization (whole animal-, tissue-, cell- and gene expression-level). We aimed to investigate effects of environmental perturbation on *L. elliptica* with a special emphasis on age-specific sensitivities, in order to gain a picture of how the species' populations are affected by current and future environmental changes.

Specific aspects were addressed:

A) In which way does *L. elliptica* respond to enhanced sediment runoff and ice scouring, and are these responses age-dependent?

Physiological responses of bivalves exposed to high turbidity or frequent ice scouring might appear on the levels of performance (burrowing activity/survival), metabolism

(respiration, regeneration capacity), on cellular levels (cellular immune responses, intracellular metal accumulation, oxidative stress leading to damage of macromolecules) or on the level of gene expression and manifest in an age-specific manner. Stress experiments under field and laboratory conditions were designed to gain a first picture of stress-induced physiological changes using a wide range of analytical levels and parameters.

On the other hand, effects on the animals' physiology may only become visible upon long-term exposure to a moderate stressor, after a history of frequent catastrophic impacts or may result from a combination of up-adding stressors. In order to look for physiological effects of distinctly differing stress levels, *L. elliptica* individuals were investigated from two field-stations of the Potter Cove (King George Island, WAP), which are characterized by different scales of turbidity and ice scouring impact.

B) Is the *L. elliptica* cellular stress- and immune response sensitive to environmental disturbance such as varying food conditions and/ or physical injury?

Another aim of this thesis was to understand physiological responses of the whole-organism (functional) and on the cellular level towards intense discharge of turbid meltwater (increasing uptake of inorganic particles, potential food deprivation) and ice scouring (injury). Therefore, effects of injury and starvation on parameters of immunity and stress response were investigated. In bivalves, a systemic consequence of environmental stress might be a suppression of capacities for stress responses, the animals rely on. Animals in areas highly exposed to different stressors might be left to be more susceptible to further increasing stress levels. This might happen in a sequential way, as cellular damage or disturbed cell signaling pathways potentially lead to a functional impairment. Indeed, environmental impact such as food (Butt et al. 2007; Hegaret et al. 2004), mechanical disturbance (Ballarin et al. 2003), high temperature (Parry and Pipe 2004; Hawley and Altizer 2011) or inorganic pollution/xenobiotics (Auffret 2005; Oliver et al. 2001) were linked to the immunological competence in bivalves. Several viral, bacterial and protozoan diseases were found to be promoted by environmental disturbances and extensive mass mortalities induced by bacterial infections are a known phenomenon in bivalves (Hawley and Altizer 2011; Beaz-Hidalgo et al. 2010; Morley 2010). However, little is known about exact mechanisms governing stress response capacities in bivalves. As hemocytes and hemolymph play important roles in the bivalves physiology, ranging

from gas exchange, osmoregulation, nutrient and waste material transport to immune functions, these cells are important mediators of external stimuli to the bivalves physiology (Gosling 2003). Beside an identification and basic characterization of the hemocyte's functions, we aimed to verify a starvation- and injury-induced impact on the *L. elliptica* cellular defense system.

C) *Are L. elliptica* components of immunity specifically responding to external stress? What are specific characteristics of the *L. elliptica* immune functions?

Beyond the estimation of effects of sedimentation and ice scouring on the bivalves physiology, a major point of interest within this thesis was the basic characterization of *L. elliptica* immune functions. Recently the interest in the innate immune system, its evolution and the role of circulating hemocytes (immunocytes) of marine invertebrates has grown. However, the immune system of *L. elliptica* as well as most other Antarctic invertebrates has not been sufficiently investigated. In order to contribute to a better understanding of the *L. elliptica* external defense, a first characterization of major immune parameters, in particular of circulating hemocytes, as well as corresponding cellular responses towards microbial stimuli was undertaken.

D) *Can one expect a population response to ongoing changes in the environmental setting/ rising stress levels?*

Correlated with the development of warming trends, multifold changes in disturbance-levels can be expected, which might have ecological consequences for benthic populations and also *L. elliptica*. We hypothesize, that the rising stress levels might lead to an altered organismic performance and fitness of the animals, which, as a consequence, will lead to a change in the *L. elliptica* population dynamics with respect to age relationship and potentially has an impact on the community structure in affected areas. By integrating a suite of whole organismic, biochemical, cellular and molecular responses of *L. elliptica* under effects of increasing turbidity, ice scouring and food restriction, particularly focusing on age-specific sensitivities, we aimed to generate a first picture of the *L. elliptica* populations' stress response.

2. LIST OF MANUSCRIPTS

This thesis consists of three first-author manuscripts which are published (manuscript I and II) or in revision (manuscript III) and one published co-authorship paper (manuscript IV). Further currently unpublished data are presented in the Additional Results.

MANUSCRIPT I

Husmann, G., Philipp, E.E.R., Rosenstiel, P., Vazquez, S., Abele, D. (2011).

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

Journal of Experimental Marine Biology and Ecology 398: 83-90.

The immune system of *L. elliptica* or most of other Antarctic invertebrates has not been investigated to date. This manuscript aims to characterize basic components of the *L. elliptica* immune system as well as immune responses towards microbial stimuli. In order to verify a potential impact of turbidity and damage due to ice scouring on a more systemic level, hemocyte (immunocyte) abundance was detected during food deprivation and injury treatments. Conception, experiments, sampling, analyses and writing were carried out by G. Husmann under supervision of E.E.R. Philipp and D. Abele. The antibacterial assays were carried out by S. Vazquez and G. Husmann. The co-authors advised on the interpretation of the data and contributed to the writing.

MANUSCRIPT II

Husmann, G., Abele, D., Monien, D., Monien, P., Kriews, M., Philipp, E.E.R. (2012)

The influence of sedimentation on metal accumulation and cellular oxidative stress markers in the Antarctic bivalve *Laternula elliptica*.

Estuarine, Coastal and Shelf Science 111: 48-59.

This manuscript was aimed to link locally high inorganic turbidity in Potter Cove, triggered by massive glacial disintegration and terrigenous runoff, to the content of selected metals as well as to the oxidative stress status in *L. elliptica*. Tissue metal concentrations of animals under distinctly different disturbance scenarios (laboratory and field) were compared to investigate whether elevated runoff and turbidity indeed lead to increasing metal accumulation under the present conditions in Potter Cove. Histological and biochemical analysis in *L. elliptica* tissues was carried out by G. Husmann. Metal analyses in bivalve

tissues were carried out by I. Stölting and G. Husmann under supervision of M. Kriews. The manuscript was written by G. Husmann in cooperation with all co-authors. Conception, field-sampling and the sedimentation treatment was carried out by E.E.R. Philipp. V. Hanke helped with the detection of metals and protein carbonyls. Environmental data (metal measurements in sediments and water) were provided by D. Monien and P. Monien.

MANUSCRIPT III

Husmann, G., Abele, D., Rosenstiel, P., Clark, M.S., Kraemer, L., Philipp, E.E.R. (under review)

Age dependent expression of stress and antimicrobial genes in the hemocytes and siphon tissue of the Antarctic bivalve *Laternula elliptica* exposed to injury and starvation.

In revision (Cell Stress and Chaperones).

This manuscript describes the characterization of a transcriptomal data set generated by pyrosequencing (454) from RNA of hemocytes and tissues of different stress treated *L. elliptica*. We further investigated effects of high turbidity (“food deprivation”) and ice scouring (“injury”) on molecular expression levels in younger and older individuals. We aimed to identify genes, which are reliable markers for the characterization of a stress/immune gene expression response in *L. elliptica*, to answer the following questions: Is *L. elliptica* starved under experimental food deprivation, and are effects different in young and old individuals? Is the ability to respond to stress (injury) affected by starvation? Conception, stress experiments, sample processing as well as molecular works and writing were carried out by G. Husmann. The generation and further analysis of the transcriptome data set was done in cooperation with L. Kraemer, E.E.R. Philipp and M.S. Clark. Statistics were carried out by G. Husmann under supervision of E.E.R. Philipp. D. Abele, P. Rosenstiel and E.E.R. Philipp advised on the interpretation of the data and contributed to the writing.

MANUSCRIPT IV

Philipp, E.E.R., Husmann, G., Abele, D. (2011).

The impact of sediment deposition and iceberg scour on the Antarctic soft shell clam *Laternula elliptica* at King George Island, Antarctica.

Antarctic Science 23: 127-138.

This manuscript describes the investigation of physiological effects of sedimentation and ice scouring on *L. elliptica* at the whole-animals level. A set of field and laboratory experiments

was carried out at Potter Cove, King George Island (WAP), comparing age-dependent activities, metabolic and survival rates in two populations under different scales of turbidity and ice scouring. The mortality experiment was carried out by G. Husmann, who also contributed to the writing. Conception, experiments, analysis and writing were carried out by E.E.R. Philipp.

3. MATERIAL AND METHODS

Animal sampling as well as field and *in situ* experiments for this thesis were conducted during two expeditions to King George Island/Isla 25 de Mayo (South Shetland Islands, Antarctica) in 2006/2007 and 2008/2009. Individuals of the Antarctic clam *Laternula elliptica* were sampled by SCUBA diving and brought to the Dallmann Laboratory at the Carlini Station (formerly named Jubany Station). Here, laboratory animals were kept in natural seawater at constant temperatures (1°C) until they were used for *in vivo* and *in vitro* stress experiments.

The majority of biochemical analysis and the histological lipofuscin detection were conducted at the Alfred Wegener Institute for Polar and Marine Research (AWI) in Bremerhaven. The molecular work and histological determination of proliferation rates was done at the Institute of Clinical Molecular Biology (ICMB), Kiel in Germany.

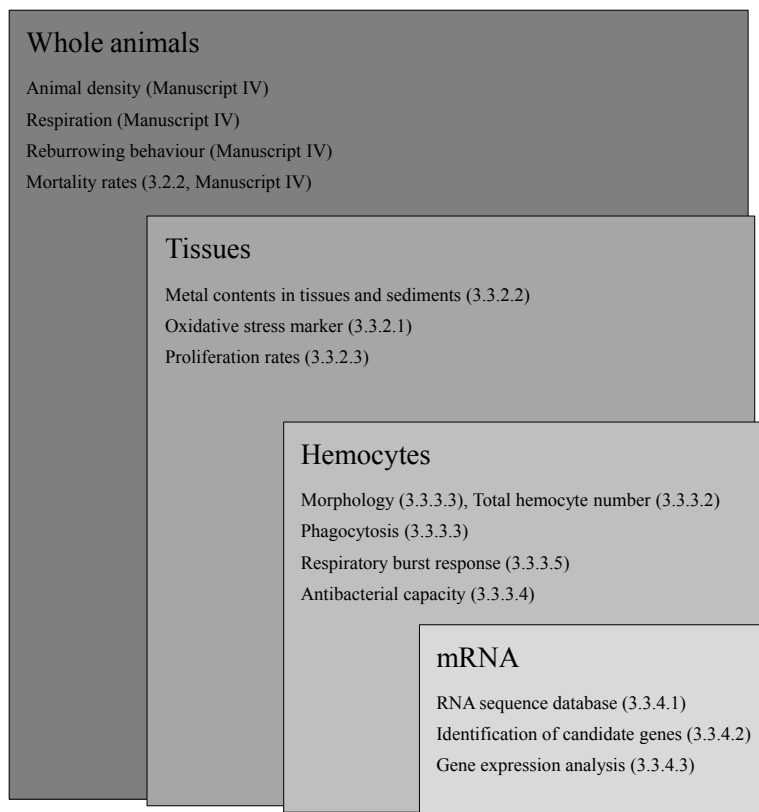


Figure 9: Diagram showing the different parameters and methods which were applied at different levels of the *Laternula elliptica* physiological organization. Approaches are further described in this section and the manuscripts.

Physiological stress responses of *L. elliptica* were characterized on various levels of organizational complexity including whole animals, selected tissues, hemocyte cells

(immunocytes) or RNA (Fig. 9). The different methods have been described in manuscripts I – IV and are only briefly summarized here.

3.1 Field station comparison/Field sampling from two *in situ* stations

In order to gain a first picture of comprehensive long-term effects, *L. elliptica* individuals were sampled directly from two populations distinctly impacted by sediment and ice scouring (L1 and L2).

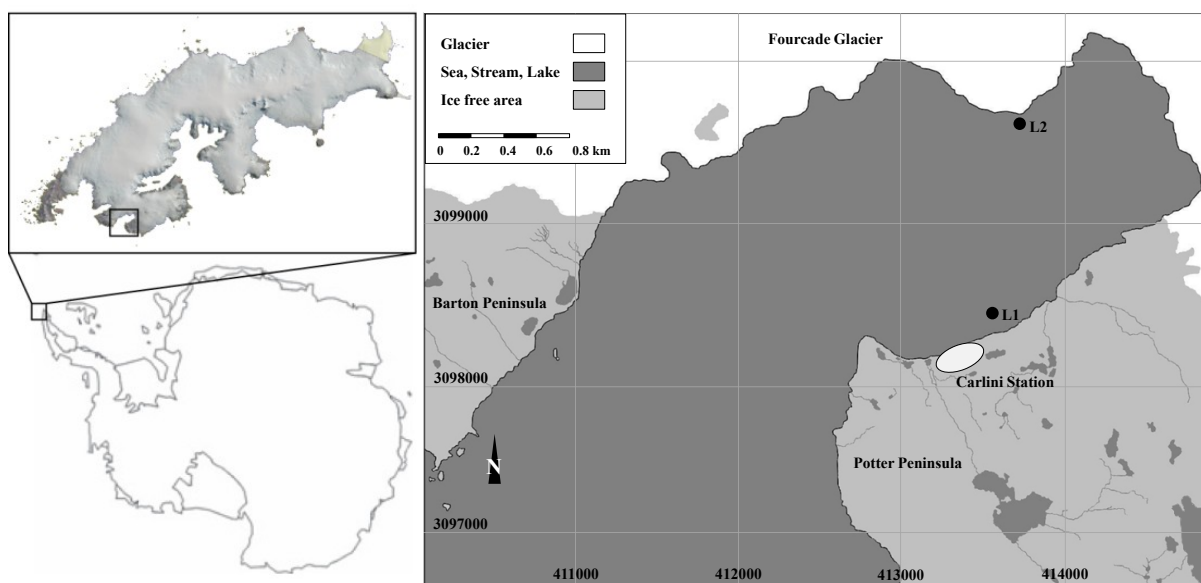


Figure 10: Maps of the location of King George Island (KGI) on the Western Antarctic Peninsula, Potter Cove located on KGI and Potter Cove. Dots mark the two sampling sites of *Laternula elliptica*. L1: “exposed” population (UTM, Z21S, 413557 E, 3098441 N), L2: “less exposed” population (UTM, Z21S, 413724 E, 3099626 N). Further the position of the Argentinean base ‘Carlini’ (former “Jubany Station”) is given. Figure 10 was drawn according to a map taken from <http://www.kgis.scar.org> (IPG, University of Freiburg; 2009).

The sampling location L1 lies within a major runoff plume of the Fourcade Glacier in Potter Cove (Fig. 10, L1: UTM, Z21S, WGS 84, 413557 E, 3098441 N), L2 is more remote from the major glacial run off area on the opposite of Carlini station (L2: UTM, Z21S, 413724 E, 3099626 N) (Klöser et al. 1994; Varela 1998). Due to the oceanographic setting, which is described by Schloss and Ferreyra (2002), a major fraction of floating ice drifts towards the “more disturbed” station. Here, grounding ice blocks are often observed (Fig. 6 and 11).

Hence, both stations also differ distinctly in terms of ice-impact. A detailed characterization of the environmental setting is presented in manuscript **IV**.



Figure 11: South eastern shore of Potter Cove: beaching brash ice and ice blocks from the disintegrating Fourcade glacier or entering the cove from the ocean.

After sampling, the animals were brought to the Dallmann Laboratory, where they were kept in flow-through-tanks with natural seawater from the cove. Subsequently, the animals were dissected and ganglia fixed for histological analysis of lipofuscin. Digestive gland, mantle and gills were shock frozen and stored in liquid nitrogen for detection of metal concentrations and protein carbonyls (**3.3.2.1**, manuscript **II**). Additional animals were sampled for experimental detection of respiration changes as well as metal accumulation at different sediment concentrations, as also described in detail in manuscript **II** (see also **3.2.1**) and manuscript **IV**. Animals were allocated to non-overlapping age classes. Approximate ages of the animals were estimated from shell size using a *L. elliptica* von Bertalanffy growth model from Potter Cove. Details of the method used are described under **3.3.1** in more detail.

Sediment samples were taken in close range of both stations using acrylic glass push corers operated by divers. Samples taken from the cores were further processed at the Dallmann Laboratory and transported to the Institute for Chemistry and Biology of the Marine Environment (ICBM, Oldenburg, Germany) where metal concentrations and ratios of total organic carbon (TOC) to total nitrogen (TN) were determined by P. Monien (3.3.2.2). Methodological details are described in manuscript II and IV, which provides a basic characterization of sedimentation rates and abundances of *L. elliptica* at both stations.



Figure 12: Sub-littoral in Potter Cove. Left side: plume of resuspended sediment, right side: marine “sediment rain”. Picture taken by R. Sahade (Cordoba University, Argentina).

3.2 Experimental setups

3.2.1 Sediment treatment

To investigate direct effects of high turbidity on tissue metal accumulation and content of protein carbonyls, *L. elliptica* (76 ± 5 mm shell length) individuals were sampled from the less impacted station (L2) and placed into either of two 180 L aquaria (Fig. 13). Over a period of 28 days, the animals of one tank were treated by $1200 \text{ mg runoff sediment L}^{-1}$ (Fig. 13B), whereas the animals of the other tank served as untreated controls (Fig. 13A). Following 28 days, the animals were dissected and digestive gland, gills and mantle shock frozen and stored in liquid nitrogen until further processing. Details of the experimental setup and maintenance of the animals are given in manuscript II.

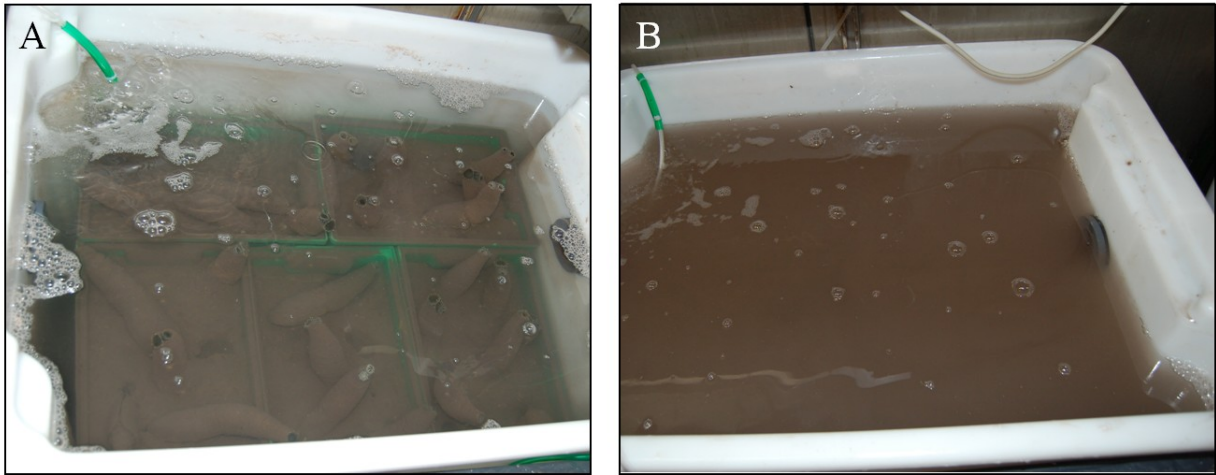


Figure 13: Sediment treatment of *Laternula elliptica*. The animals were treated over 28 days with 0 (A) and 1200 mg sediment L⁻¹ (B).

3.2.2 Mortality experiment

The susceptibility of *L. elliptica* to a variety of injuries was investigated in different size/age classes. Following the approach of Ziuganov (2000), 3 types of wounds were inflicted to the animals: siphon cuts, adductor muscle cuts and shell cracks (N = 12 per type of injury and size class). The animals were allowed to recover in four 180 L tanks refilled with natural seawater on a daily basis, constantly aerated and maintained in a temperature constant room. Mortality rates under each type of injury was determined for a period of 38 days; not injured animals served as untreated control (N = 12 per size class). Further details and results are presented in manuscript IV.

3.2.3 Stimulation *in vivo*: Stress-experiment “injury and starvation”

To investigate, whether starvation has an impact on the stress responses of *L. elliptica* under injury, the animals were separated into two non-overlapping size classes (Fig. 14). Younger and older individuals were subjected to starvation, high nutrient loads and injury. The animals were kept in 4 separate tanks (40 young and 40 old animals per tank) with filtered seawater (10 µm final pore size) without sediment. 10 days after acclimation half of the animals were subjected to finer filtered seawater using a system of a sieve pre-filter (pore size of 200 µm) and polypropylene cartridge filters (Wolftechnik Filtersysteme, Weil der Stadt, Germany) to a final pore size of 0.5 µm (treatment “starvation”). The other half of animals (treatment “food”) was kept in unfiltered seawater and every second day fed *ad libitum* using a mixed diet of live microalgae as well as “artificial detritus”: freeze dried and grinded macroalgae (*Ascoseira mirabilis*), krill and red bloodworms (Tetra, Melle, Germany). The live

phytoplankton was obtained from Potter Cove using plankton nets. It was grown in natural seawater under light for several days before fed to the animals. After 21 days half of the individuals of each size group were injured in such way that the mantle was injured by cracking both halves of the shell and the siphon was cut at two places (Fig. 14). Animals were sampled after two and 21 days of recovery from the injury event (i.e. on day 23 and 44 after starting the nutrition treatments). Hemocyte samples were taken and the animals were dissected. Hemocyte pellets and tissues (digestive gland, gills and siphon) were immediately shock-frozen in liquid nitrogen for RNA expression analysis (3.3.3.1, 3.3.4). Following hemolymph sampling, a fraction of the collected hemolymph was immediately diluted with Alsever's anticoagulant solution for hemocyte counts (HC). A detailed description of the holding system and the experimental setup as well as the methods used is presented in manuscript I and III, the procedure of hemocyte sampling for hemocyte counts is described under 3.3.3.1 and 3.3.3.2.



Figure 14: Animals with injuries inflicted to shell, mantle and siphon.

3.3 Sample analysis

3.3.1 Age determination

Individual ages of *L. elliptica* can be inferred from morphological features of the shell such as shell length (Fig. 15) (Brey et al. 2011; Philipp et al. 2008a). In the present study, the approximate individual ages of the animals were inferred from a von Bertalanffy growth model (VBGM), which describes the length-at-age relationship and was fitted to length-at-age data received from 553 animals from Potter Cove (Philipp et al. 2008a).

$$S_t = 90.08 * (1 - e^{-0.109 * (t+1.665)})$$

S_t = shell length [mm]

t = individual age [yrs]

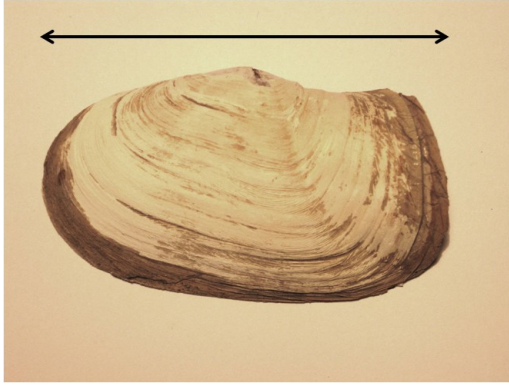


Figure 15: Shell length determination (arrow) of *Laternula elliptica*.

3.3.2 Biochemical and histological techniques

3.3.2.1 Analysis of oxidative damage parameters

Detection of protein carbonyls

Protein carbonyls were quantified in homogenates of mantle tissues according to Levine et al. (1990) and as described for *L. elliptica* in Philipp et al. (2005a). Carbonyl groups are introduced by the irreversible oxidative modification of protein side chains during several oxidative pathways. The formation is catalyzed by ROS or resulting from secondary reactions with oxidized non-protein components. Thus, the presence of protein carbonyls has been widely used as biomarker of protein oxidation upon oxidative stress (Catalgol et al. 2012). The assay used for protein carbonyl quantification is based on the derivatisation of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), leading to the formation of a stable dinitrophenylhydrazone-protein conjugate, quantified spectrophotometrically at 360 nm. To relate the concentration of protein carbonyls to the protein content, the protein concentration was detected for each sample using the Bradford method (1976). A detailed description of the method is presented in manuscript II.

Analysis of lipofuscin

Lipofuscin is a stable end product of lysosomal oxidatively degraded cellular components which is accumulating intracellularly. As the only known mechanism to remove the autofluorescent and not further degradable polymeric material is cellular proliferation (Terman and Brunk 2004), presumably postmitotic ganglia (Fig. 16) were sampled from *L. elliptica* for the determination of lipofuscin content [% area fraction] using a Leica TCS NT confocal laser scanning microscope (excitation: 488 nm, emission: 521 nm long pass filter). Details of the techniques used for processing and analysis of the samples are described in manuscript II.

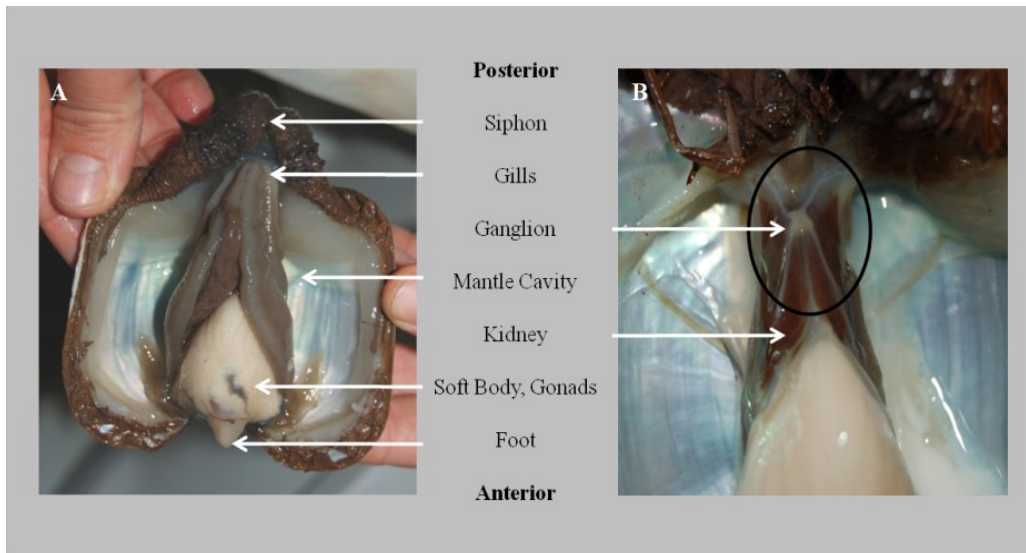


Figure 16: *Laternula elliptica* dissection: **A:** Ventral view into the open shell (mantle cut along the mantle edge, which is completely fused in these clams). Ingested and accumulated material is visible in the gills. **B:** Same perspective with gills removed to expose the ganglion as well as connected nerves. Picture taken by: E.E.R. Philipp.

3.3.2.2 Metal concentration in tissues and sediment

The concentrations of cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), strontium (Sr), vanadium (V) and zinc (Zn) were investigated in gills, digestive gland and mantle of *L. elliptica*. Values were assessed as $\mu\text{g g}^{-1}$ dry weight (dwt). The detection was carried out by I. Stölting (AWI) using inductively coupled plasma optical emission spectrometry (ICP-OES, IRISb Intrepid Type Duo, Thermo Nicolet GmbH). Sample procession and detection method are described in detail in manuscript II.

Samples of the sedimentary environment were ground and concentrations of major and trace elements (Al_2O_3 , Fe_2O_3 , MnO, Cu, Sr, V, Zn) detected using a conventional wavelength dispersive X-ray fluorescence (WD-XRF) spectrometer (Philips PW 2400). Concentrations of Fe, Mn and V in water samples from Potter Cove were detected by inductively coupled plasma mass spectrometry (ICP-MS, Elements 2, Thermo Scientific). Environmental data (metal content in sediments and water) were provided by D. Monien and P. Monien (ICBM). A detailed description of the methods used for metal measurements in sediments and water is presented in manuscript II.

3.3.2.3 Proliferation rate analysis in outer epithelia of different tissues and in gills

Cell proliferation rates were assessed by BrdU staining of dividing nuclei, using a modified protocol after Moore et al (1994). The main principle of the method consists in the

incorporation of the thymidin analogon bromodeoxyuridine (BrdU) into replicative DNA of dividing cells. Intracellular thymidylate synthase is inhibited by addition of fluorodeoxyuridine (FdU), therefore FdU reduces the competition between endogenous thymidine and its analogon BrdU, thereby increasing BrdU incorporation.

L. elliptica individuals were hand collected by divers in Potter Cove after the Austral winter (winter animals, 6th November 2008) and during late Austral summer (summer animals, 5th March 2009) in front of Carlini Station. Animals were classified as “old” with (mean and SD) 80 ± 5 mm shell length and “young” with 46 ± 5 mm shell length. For BrdU labelling, the different sized animals were kept in individual flasks containing filtered, aerated seawater at constant temperatures of 1°C in the dark and without nutrition. 5-Bromo-2-deoxyuridine (Fluka, 30 mg L^{-1}) and 5-Fluoro-2-deoxyuridine (Fluka, 3 mg L^{-1}) were added for bath-labelling. Every 24 hours half of the water was replaced by fresh seawater containing appropriate concentrations of BrdU and FrdU in order to ensure a good water quality and maintain constant concentrations of both chemicals. After 5 days of bath-labelling the animals were dissected, and samples of different tissues (gill, mantle, siphon, muscle and foot) transferred to cyrovials and shock-frozen in liquid nitrogen for storage and transport. At the ICMB in Kiel, BrdU-labelled tissues and tissues of non-labelled animals (controls) were embedded for cryo sectioning (Jung Tissue Freezing Medium, Leica Microsystems) and cut to $6 \mu\text{m}$ sections using a Cryostat (CM 1850, Leica Microsystems) at -22°C . Cryosections were placed on microscopic slides (Superfrost Plus, Thermo Scientific), fixed in dry-ice cooled acetone (Merck) for 10 minutes and frozen at -80°C until further processing. Slides were stained using biotinylated mouse anti-BrdU antibody, taken from a BrdU In-Situ Detection Kit (BD Pharmingen), as well as a secondary antibody and 3,3'-diaminobenzidine (DAB), see below. Prior to immunohistostaining slides were warmed to room temperature and dipped in phosphate-buffered saline (PBS, 2-times for 2 minutes). Slides were then incubated in 2% hydrogen peroxide for 15 minutes to deactivate endogenous peroxidases and washed again in PBS (3 times for 2 minutes). In a humid chamber, sections were covered with bovine serum albumin solution (0.75% in PBS) for 20 minutes. The BrdU antibody was diluted 10-fold and sections incubated for 2 hours. After 3 washing steps with PBS (each 2 minutes) sections were covered with a biotinylated secondary antibody connected to a peroxidase system from a Vectastain Elite ABC Kit (Vector Laboratories, USA) and incubated in a humid chamber for 45 minutes, followed by 2 washing steps in PBS (each for 5 minutes). DAB staining reaction was performed with a Peroxidase Substrate Kit (Vector Laboratories, USA) and stopped in PBS (2 times) after development of a visible brown-black staining. A second section of each

sample was processed similarly but covered with PBS instead of the primary antibody to serve as a control of the method. To visualize cell nuclei without incorporated BrdU, sections were lightly stained with hematoxylin. Analysis and digital imagery of the stained sections was performed using an Axio Imager.Z1 microscope (Carl Zeiss) combined with an Axiocam digital camera (Carl Zeiss) and a 63x objective equipped with an Axiovision Release 4.7.1 software (Carl Zeiss). Analysis of 10-15 images per section and individual bivalve was carried out using “ImageJ” software (National Institutes of Health, USA, <http://rsbweb.nih.gov/ij>). Averages of the amount of nuclei (N) with incorporated BrdU (N_{BrdU}) to the total count (N_{total}) of stained nuclei were calculated in order to obtain proliferation rate $_{5 \text{ days}} [\%] = N_{\text{BrdU}}/N_{\text{total}} \times 100$. Proliferation rates could be analysed in mantle, siphon, posterior adductor muscle, foot (outer epithelia respectively) and gill (N=5-6 per group).

3.3.3 Hemocyte characterization

For the first time, hemocytes of *L. elliptica* were isolated *ex vivo* and basic physiological functions investigated by simple stimulation experiments. Investigation of general morphological features (i.e. aggregation response, phagocytotic activity), respiratory burst response to external stimuli, or antimicrobial properties can give hints to common functions of invertebrate hemocytes such as digestion, transport, excretion of antimicrobial peptides, tissue repair and defense responses.

3.3.3.1 Hemolymph extraction/hemocyte sampling

For hemocyte sampling, the covering periostracum was removed from the accession to the posterior adductor muscle. Afterwards the hemolymph containing the hemocyte cells was collected. A 16-gauge needle with a 10 ml-syringe was inserted into the posterior adductor muscle and the hemolymphatic fluid slowly withdrawn from the open circulatory system. Between 3 and 6 ml of hemolymph was sampled per animal, depending on the individuals' size. The hemolymph was centrifuged immediately and the hemocyte pellet shock-frozen in liquid nitrogen for mRNA investigations (for details of the method for RNA extraction from hemocytes see **3.3.4.3** and manuscript **III**) or immediately used for stimulation experiments or hemocyte counts (this section and manuscript **I**). To avoid allogenic reactions during stimulation experiments, hemolymph samples of different individuals were never pooled. For the same reason, hemocytes were not separated from the serum, and not transferred to cell culture media, neither for stimulation experiments using microbe-associated molecular pattern

(MAMPs) as well as by using living or heat-killed natural bacteria (3.3.3.3, 3.3.3.5), nor for optical hemocyte characterization (3.3.3.3).

3.3.3.2 Hemocyte counts

Hemocytes are not restricted to the open circulatory system (hemolymph), can move to organs, connective tissues or wound inflicted sites. Variations in number of free floating hemocytes (HC) have been widely used as a marker of the stress response in invertebrates.

Hemolymph samples of *L. elliptica* were constantly inverted to avoid adherence, diluted in Alsever's anticoagulant solution and immediately used for hemocyte counts (HC). The cell density was assessed using a hemocytometer (Neubauer improved counting chamber), and hemocyte densities calculated as cells per ml. A detailed description is presented in manuscript I.

3.3.3.3 Optical characterization of hemocytes

Hemocytes were allowed to adhere to cooled microscopic slides and basic features, such as general morphology, phagocytic activity or response to microbial mimetics investigated by light microscopy *in vitro*. Different conserved and non-self molecules (microbe-associated molecular pattern, MAMPs) as well as living and heat-killed natural bacteria from Potter Cove were applied for the stimulation: Lipopolysaccharide (LPS, research center Borstel), peptidoglycan (PGN, Biochemika 77140), phorbol-12-myristate-13-acetate (PMA, Biochemika 79352), flagellin (Invivogen), zymosan (Sigma Z-4250) as well as synthetic oligonucleotides (ODN2006 – type B, Invivogen). Natural bacteria were isolated from the Potter Cove environment (seawater), as well as directly from the animals and their sedimentary environment. A detailed description of the morphological hemocyte characterization by light microscopy is presented in manuscript I.

3.3.3.4 Antibacterial assay

L. elliptica hemolymph and gills were investigated for antibacterial properties. Two assays were performed using bacterial mixtures which were isolated from the Potter Cove water column or the animals' sedimentary environment. For assay (A) pooled samples of native hemolymph and homogenated gills were applied to sterile filter stripes. Stripes were placed on marine agar plates streaked with lines of isolated bacterial morphotypes, and the inhibition of colony growth observed. For assay (B) bacterial mixtures isolated from the cove in suspension were mixed with samples of hemolymph and homogenated gills. The mixtures

were plated onto marine agar and observed for colony growth (a detailed description of the sample processing is presented in manuscript I).

3.3.3.5 Nitroblue tetrazolium (NBT)-reduction assay

The generation of ROS was measured in hemocyte suspensions (native hemolymph) either unstimulated or stimulated by MAMPs as well as by living or heat-killed natural bacteria from Potter Cove (3.3.3.3). ROS such as $O_2^{\cdot-}$, NO and presumably other ROS such as peroxynitrite ($NOO\bullet$) are reducing the soluble, yellow colored dye nitroblue tetrazolium (NBT), leading to the formation of blue NBT-formazan deposits, which can be quantified spectrophotometrically. The optical density was detected at 620 nm using a TRISTAR microplate reader (Fa. Berthold, Germany) and normalized to hemocyte numbers in the sample. Details of the method are presented in manuscript I.

3.3.4 Molecular Biology

3.3.4.1 Generation of the RNA sequence database

A RNA sequence data set was generated using hemocytes and tissues (gills, digestive gland and siphon) sampled from *L. elliptica* of both age groups and different treatments of the stress-experiment “injury and starvation” (3.2.3). This procedure was chosen to allow for a broad spectrum of mRNAs, transcribed under stress conditions and in two distinct life stages, for sequencing and subsequent annotation.

Total RNA was extracted from frozen samples of tissues as well as from a pool of hemocyte pellets. Details about sampling of hemocytes and tissues for RNA isolation are given in manuscript III.

mRNA was extracted from a pooled sample, composed of equal amounts of the extracted total RNA. Following a spectrophotometrical quality check, the mRNA was used for cDNA generation. cDNA was sequenced on the Genome Sequencer FLX system (454 Life Sciences, Branford, CT, USA) done by the sequencing platform of the ICMB using Titanium chemistry according to the manufacturers’ protocol.

M. S. Clark of the British Antarctic Survey (BAS) provided an additional set of expressed sequence data, derived from mantle tissue of untreated *L. elliptica* sampled at Rothera Station (WAP) (Clark et al. 2010). In subsequent steps both 454 datasets were processed bioinformatically with the help of L. Kraemer (ICMB). Sequences were cleaned from polyA tails, smart primer sequences, 454 adapter sequences or short read fragments. The remaining sequences of the first (ICMB) and second (BAS) dataset were assembled to the final contig

dataset. A detailed description of the biological samples used as well as the methodological strategy of mRNA sequence dataset generation (Fig. 17) is presented in manuscript III.

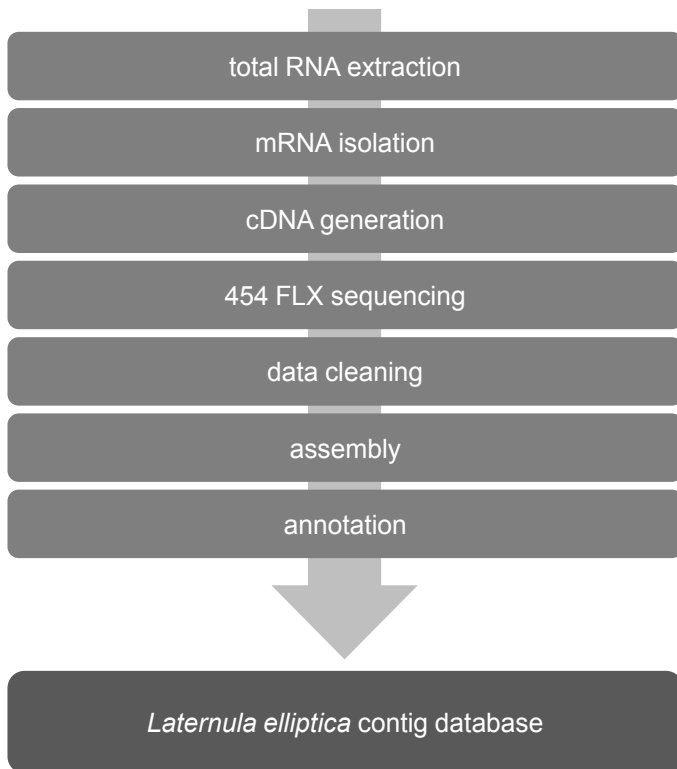


Figure 17: Schematic overview of the strategy for the *Laternula elliptica* stress response mRNA sequence dataset generation

3.3.4.2 Identification of candidate genes

Putative gene names were assigned to the assembled contigs by BLASTx analysis against the UniprotKB/Swissprot protein database, and the tBLASTx against the NCBI non-redundant nucleotide (nt) database. Conserved protein domains were identified using InterProScan (Hunter et al. 2009). Sequence data together with annotation data were loaded into T-ACE, a software tool for procession of large RNA sequence data of non-model organisms (Philipp et al. 2012a).

Candidate genes potentially involved in metabolic-, injury-, immune- and general stress responses of *L. elliptica* were identified by key word search and protein structure in the annotated database.

Candidate sequences were manually re-blasted against the NCBI nr/nt and UniProtKB/Swissprot database using tBLASTx and Blastx, respectively. The protein domain structure of the different contigs was re-checked by the NCBI conserved domain architecture retrieval tool cDART and compared to the conserved domain structure for the respective gene

as defined by HomoloGene. Further details and online sources of the tools used for annotation are described in manuscript **III**.

3.3.4.3 Quantitative *real time* PCR (qRT-PCR)

In order to compare expression levels of selected genes, quantitative *real time* PCR was performed using template cDNA which was in the first line generated from samples of the experiment “injury and starvation” (**3.2.3**). Details about sampling of hemocytes and tissues for RNA isolation are presented in manuscript **III**. The RNA of each individual animal and sample was extracted separately using TRI Reagent (Sigma-Aldrich, St. Louis, USA) according to Clark et al. (2010) using minor modifications. For RNA extraction, tissues were ground in liquid nitrogen. Tissue samples (~50 mg) or hemocyte pellets of individual animals were homogenized in 600 μ l TRI Reagent by vortexing and performing the Qiagen QiaShredder protocol (Qiagen, Hilden, Germany). 120 μ l chloroform were added to the sample, which then was vortexed, followed by centrifugation at 15500 g (15 min. at 4°C). The aqueous top layer was transferred to a fresh tube. Now 150 μ l isopropanol, 75 μ l sodium citrate (0.8 M) and 75 μ l sodium chloride (1.2M) were added. The sample was again centrifuged (15500 g, 10 min. at 4°C) and the pellet cleaned by adding 300 μ l ethanol (75%) and centrifugation (1 minute, 15500 g at 4°C). The ethanol was removed and the pellet resuspended in RNase-free water. The RNA was further purified by column cleaning in combination with DNase digestion using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). The RNA was eluted in RNase-free water and kept at -80°C until further analysis.

Total RNA of digestive glands and gills was extracted only using the Qiagen RNeasy kit after homogenization in RLT-lysis-buffer (600 μ l) and using Qiagen QiaShredder columns (Qiagen, Hilden, Germany). The quality and quantity of the extracted mRNA was checked photometrically using a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). Only samples showing 260/280 nm absorbance ratios of >2.1 were used for cDNA generation with the Advantage RT-for PCR Kit (Clontech, Heidelberg).

Table 1: Primer sequences for quantitative *real time* PCR analysis of selected candidate genes potentially involved in metabolic response, immune functions or general stress response processes. Accession numbers of the *Laternula elliptica* RNA sequence database (SRA study ERP001323, ENA project number PRJEB33), reverse and forward primer sequences and amplicon length are given for each candidate gene.

Gene name	Accession number	Amplicon length [bp]	5' → 3' forward primer 5' → 3' reverse primer
18S rRNA	HE804712	352	CACCACCAACCACCGAAT AAGACGAACGACAGCGAAAG
Actin	HE804713	375	GCCAAATCCAGACGAAGG TGAAGCCCAGAGTAAGAGAGG
Catalase	HE804714	378	CATTCTCTCCCGCACACAT GCCTTTCACGCTCCTCTG
Chitinase	HE804715	223	TGCCAAAAGAAAATGCTGATT GCCGTTGTTGAGTGTGCTAA
Heat shock protein 70 kDa (HSP70)	HE804716	394	TCTCTCACCTCCAACCTTCTT TCCATCAAACACAATCTTCG
Cu/Zn Superoxide dismutase (SOD)	HE804717	305	ACACCCAACGACTACCATCC CGGCTACCACATCCACAAG
Theromacin	HE804718	337	GCAGCCCACAGAGCAGTAAC TAAAAGCGGACACCACCAT
Tissue inhibitor of metalloproteinase (TIMP)	HE804719	322	CTGGGATTGCTTGCTGTGTA TCTGGGTCTGGATGAAAACA
Amylase	HE820924	404	CCAGTTTTCTCCCCAGGTTT ATGACTACAGCAGCCCCAAT
Cyclin B	HE820925	497	CAGCAACTCTTCCCAACCAT TCATCAACACCTCCATTTC AAG
Cyclin D	HE820926	296	ATGGATTTACTGTGTGCGGAGT CTGAAGTCTCGTTCTGGGAGT
AMP activated protein kinase (AMPK)	HE820927	404	TCCCTGACTACCTGAACAAAGA CAAGATGTGCGAATGACG
Cathepsin B	HE820928	335	TCCATTTTCTGTTCCCATC GCTGCCTCCCCTACACTATC
Ferritin	HE804761	325	TGATAGCCTCCACCTGTTCC GCCTTCTTCTTTGACCGTGA
Beclin1	HE820929	452	ATTGGCTCATTGGTCTGTTT TGGTCGCTTTTCTTGATTGTC

Primers for qRT-PCR targeting candidate genes (Tab. 1) were designed using Primer3 (Rozen and Skaletsky 2000) and OligoAnalyzer 3.1 (Integrated DNA Technologies, USA). Further details about the methods used are presented in manuscript III.

To assure primer specificity and correct contig assembly, semi-quantitative PCR was performed with GoTaq polymerase (Promega). The PCR reactions consisted of 4 µl Green GoTaq Reaction Buffer (5x, Promega), 13.3 µl H₂O, 0.5 µl of forward and reverse primer (10 µM) respectively, 0.5 µl dNTP mix (10 mM each), 0.2 µl polymerase and 1 µl template cDNA. The PCR conditions were chosen as follows:

1. Initial Denaturation:	2 minutes	94°C
2. Denaturation:	30 seconds	94°C
3. Annealing:	30 seconds	*
4. Elongation:	**	72°C
5. Repeat steps 2-4 (25-40 cycles)		
6. Final elongation:	5 minutes	72°C

Annealing-temperature (*) was depending on the melting temperature of the used primer pair, elongation-time was depending on the length of the amplified PCR fragment. ** estimated by the synthesis rate of the *Taq* polymerase (1000 bp minute⁻¹).

Presence and size of PCR products was confirmed via agarose gel electrophoresis. The DNA was isolated from the gel with the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and Sanger sequenced using Big Dye Terminator chemistry (Applied Biosystems, USA). Resulting Sanger sequences were assembled with the respective contig from the transcriptome using Sequencher version 4.5 (GeneCodes, Ann Arbor, MI, USA).

Expression changes of selected candidate genes were investigated by quantitative *real time* PCR (qRT-PCR) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) with SYBR Green (Power SYBR Green PCR Master Mix, Applied Biosystems, USA). The PCR reaction consisted of 4.5 µl Power SYBR Green PCR Master Mix, 0.25 µl of forward and reverse primer (10 µM) respectively and 5 µl template cDNA (10 ng). qRT-PCR for each sample was performed in triplicates. The conditions were as follows:

- | | | |
|--------------------------------|------------|------|
| 1. Initial Denaturation: | 2 minutes | 50°C |
| 2. Denaturation: | 10 minutes | 95°C |
| 3. Annealing: | 15 seconds | 95°C |
| 4. Elongation: | 1 minute | 60°C |
| 5. Repeat steps 2-4 (45 times) | | |

To check for artifacts, a melting curve was generated for each primer pair. The comparative CT method (delta Ct) for the relative quantification of gene expression was used (Livak and Schmittgen 2001) and data were normalized using 18s ribosomal RNA (18s rRNA) or Actin as reference gene, which itself was not found to be affected by treatment or tissue.

3.4 Statistics

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (Graphpath Software, USA). For manuscript **I** data were tested for normality and differences between two groups were evaluated by t-tests. Analysis of variance (ANOVA) was used to look for differences of hemocyte numbers in the life animal experiment between the injury, starvation and food groups compared to the control individuals.

For manuscript **II**, significant differences were evaluated performing the non-parametric Mann Whitney U-test. For manuscript **IV**, slopes of the respiration experiment were compared using linear regression analysis, and slopes and intercepts analysed for significant differences (ANCOVA). Respiration responses were compared for differences by the Johnson-Neyman technique as described in manuscript **IV**. Data for suspended sediments, IP frequencies and durations were analysed using t-test. In case of three or more groups, differences were analysed with ANOVA and Tukey post-hoc test (Kruskal Wallis ad Dunn's Multiple Comparison in case of non-Gaussian distribution). Survival curves were analysed using the Kaplan-Meyer survival curve analysis of the Graph Pad Prism software. A more detailed description is presented in the manuscripts respectively.

Statistical analyses of differences among stress treatments in manuscript **III** and **Additional Results** were performed using the software package STATISTICA for Windows version 7.1 (StatSoft Inc., USA) as well as the software GraphPad Prism. Gene expression data sets were tested for Gaussian distribution using the Kolmogorov-Smirnov-test. Homogeneity of variances was assessed by the Brown-Forsythe-Test for equal variances. When necessary, data were transformed (square-root, log, LN or $-1 * \log Y$) to achieve normal distribution and homogeneity of variances.

In case of normally distributed data statistical analysis was performed using STATISTICA. Effects of time and treatment on gene-expression were evaluated by two-way-ANOVA and the Bonferroni post-hoc test.

In case of non-normally distributed data these were analyzed using the non-parametric “adonis” PERMANOVA (Permutational Multivariate Analysis of Variance using distance matrices) (Anderson 2001) of the R software package “vegan” (Dixon 2003; Oksanen et al. 2011), R statistic software version 2.13.1.

Data sets which did not show equal variances were analyzed performing t-tests corrected for non-equal variances (Welch correction) offered by the Software GraphPad Prism. Tissue-specific differential expression of theromacin was determined by one-way-ANOVA after assessing the homogeneity of variances using Bartlett’s test.

Tissue-specific differential expression of theromacin, ferritin, cyclin B and cyclin D was determined by one-way-ANOVA after assessing the homogeneity of variances using Bartlett’s test.

Significant differences between gene-expression levels in digestive glands of older *Laternula elliptica* collected after the Austral winter and during late Austral summer were evaluated performing the non-parametric Mann Whitney U-test.

MANUSCRIPT I

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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.

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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 benthic-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 symbiotic 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^-) 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. elliptica* 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 ($62^{\circ}14'S$, $58^{\circ}40'W$), Antarctic Peninsula, between November 2008 and February 2009 at 10–15 m depth, 33.5 PSU and $1.5^{\circ}C$. 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 $1^{\circ}C$ 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 ($0.2 \mu m$) 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 $\mu g/ml$], zymosan [10 mg/ml], peptidoglycan [200 $\mu g/ml$], 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 ($1^{\circ}C$) 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 ($0.22 \mu m$ pore size). Seawater samples were collected from 10 m water depth in Potter Cove and also filtered over $0.22 \mu m$ 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 $10^{\circ}C$ for two days. Bacteria were collected by centrifugation (2300 g, 15 min, $4^{\circ}C$) 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 μ l) 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 $^{\circ}$ C), hemocytes were observed by light microscopy (Zeiss Axioskop) with 40 \times and 100 \times 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 tetrazolium (NBT)-reduction assay after Anderson et al. (1992) and Arumugam et al. (2000). The assay measures O_2^- , NO and presumably other ROS such as peroxynitrite (NOO $^{\bullet}$) 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 μ l 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 μ l of stimulant solution (microbial mimetic) or sterile seawater for controls and with 550 μ l NBT solution (0.1% in sterile seawater, 0.2 μ m filtered and kept on ice). Stimulated and control tubes were incubated for 60 min at 3 $^{\circ}$ C in the dark and inverted every 15 min to ensure mixing. After incubation, the samples were centrifuged (400 g, 2 $^{\circ}$ C, 5 min), supernatants discarded and pellets re-suspended in 550 μ l methanol (70%). After a second centrifugation (400 g, 2 $^{\circ}$ C, 5 min) supernatants were discarded again and pellets re-suspended in 350 μ l 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 $^{\circ}$ C, 20 min) to remove cellular debris, the optical density (OD) of the supernatant was recorded at 630 nm in triplicates of 100 μ l 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 μ l, 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 $^{\circ}$ 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 μ g/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 μ l 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 μ m final pore size) without sediment and maintained at a constant temperature of 1 $^{\circ}$ C and fully aerated. After 10 days of acclimation the experiment was started: half of the animals were subjected to filtered seawater (0.2 μ m 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

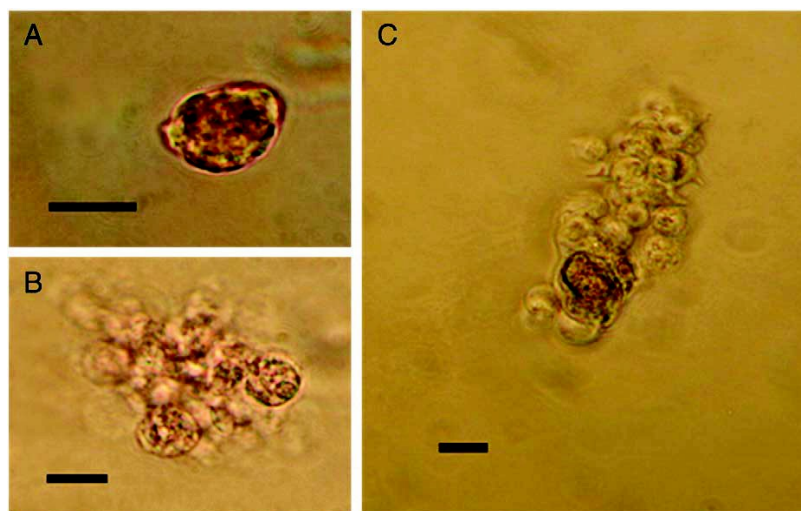


Fig. 1. Hemocytes of *L. elliptica*: (A) optically dense, larger cell type 1; (B) spherical hemocyte, transparent and with less or without granula and inclusions (cell type 2); (C) both cell types forming cluster. Scale bar = 10 μ m.

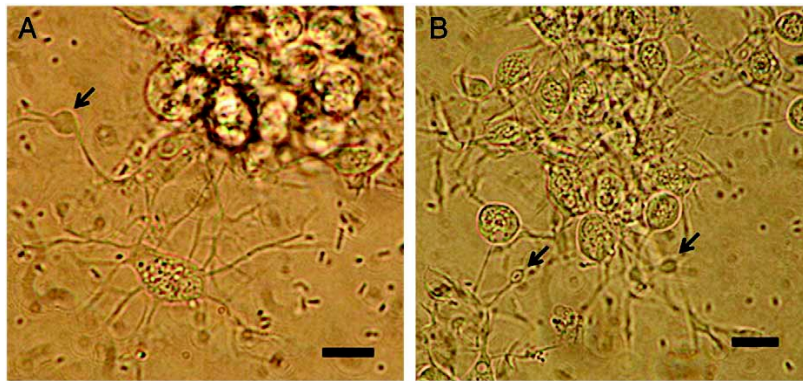


Fig. 2. Hemocytes of *L. elliptica*: Fresh preparations, unfixed. Hemocytes formed aggregates (A) and single cells, mostly of the less granular type 2, spread out on the microscopic slide generating filopodia (A and B) with visible swellings due to plasma flow (arrows). Scale bar = 10 μm .

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 protoplasm 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

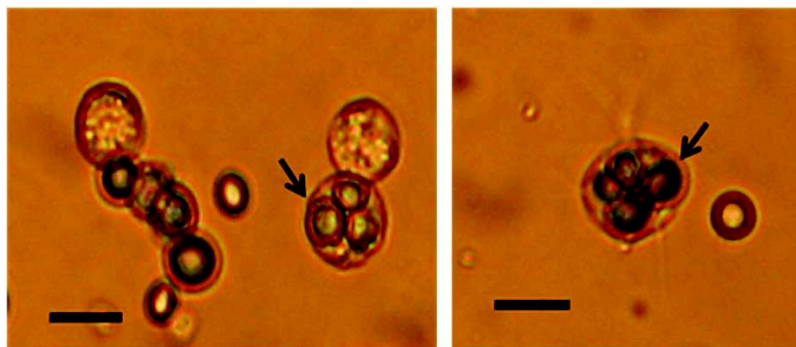


Fig. 3. Hemocytes of *L. elliptica*. Hemolymph was mixed and incubated with yeast cells (stained with bromphenol blue). Some hemocytes showed phagocytic activity and engulfed yeast cells (arrows). Scale bar = 10 μm .

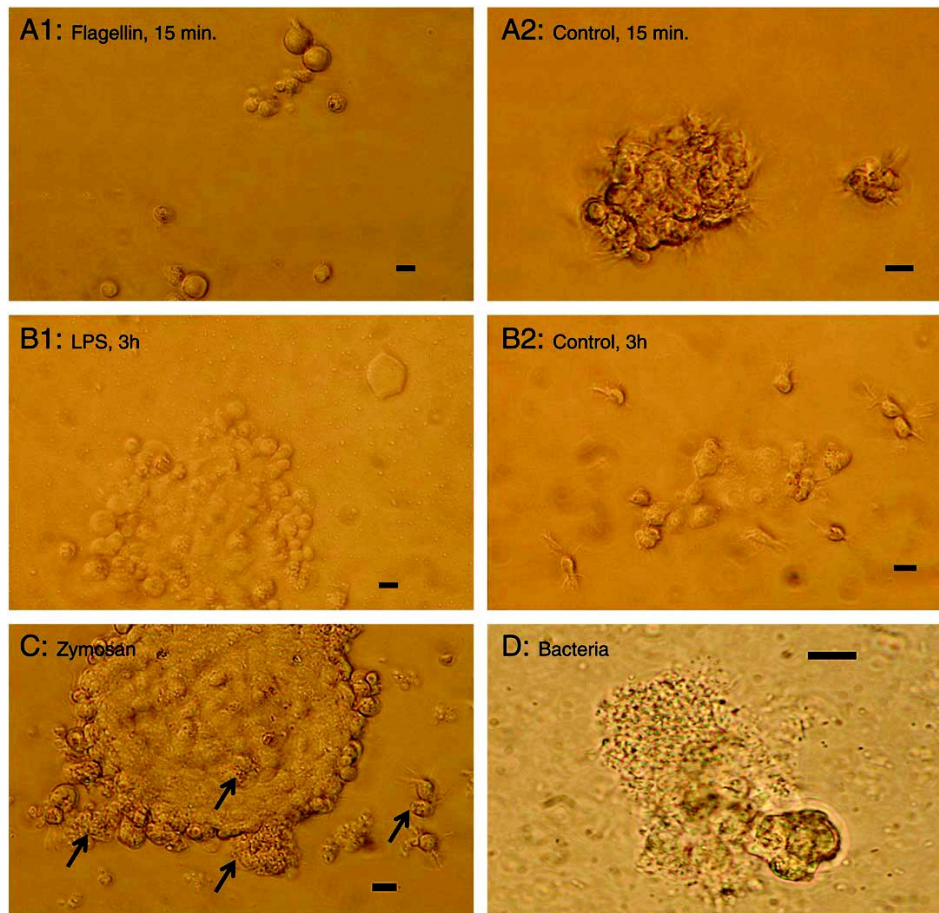


Fig. 4. Hemocytes of *L. elliptica*. Hemolymph incubated with different microbial mimetics and with bacteria isolated from Potter Cove seawater and sediments. (A1) Incubation with flagellin (15 min): less aggregates, less extension of filopodia than seawater controls (A2, 15 min). (B1) Incubation with 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 μ m.

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 phagocytosing 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, phagocytosed 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 \pm 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*
\pm 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 \pm S.D. from 6 determinations using hemocytes (size range 6.5–7 μ m) 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).

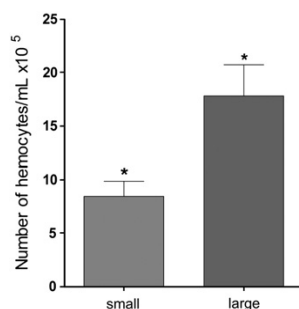


Fig. 5. Hemocyte counts (cells/ml) of small and large untreated *L. elliptica* individuals. Values are mean \pm S.E.M., $N = 6-7$ individuals per group. * = significantly different between groups, $p < 0.05$ (Student t-test).

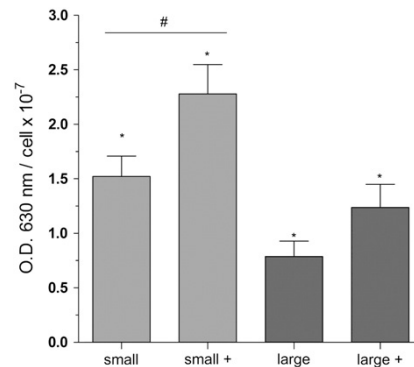


Fig. 6. Hemocyte ROS generation (formazan formation) of different *L. elliptica* size groups, untreated and in response to zymosan (+). Values are mean \pm S.E.M., $N = 5-6$ individuals per group and treatment. * = significant difference between size groups within the respective treatment, # = significant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

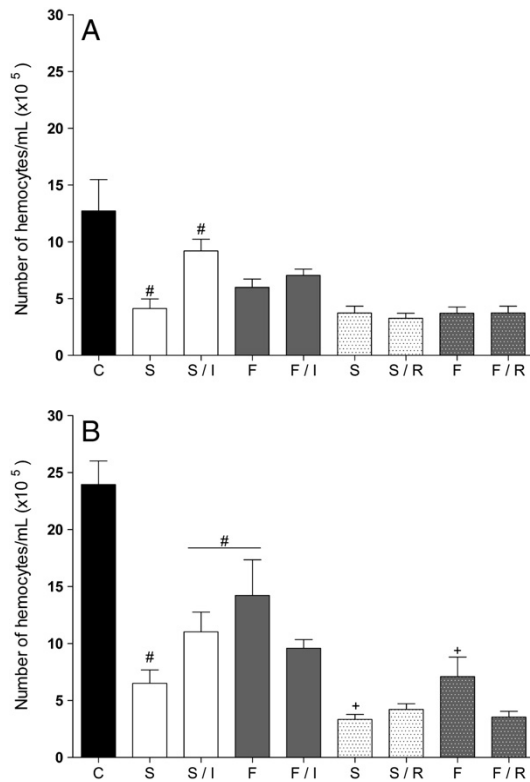


Fig. 7. Hemocyte counts (cells/ml): small (A) and large (B) *L. elliptica* individuals were sampled after 10 days of laboratory acclimation (C, black bars), 23 days under “food” (F, grey bars) and “starvation” (S, white bars) conditions and acute (2 days since infliction) injury (S/I and F/I), as well 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 \pm 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 (Hégaret 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 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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MANUSCRIPT II

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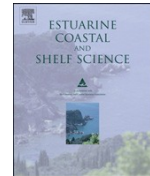
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The influence of sedimentation on metal accumulation and cellular oxidative stress markers in the Antarctic bivalve *Laternula elliptica*

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ABSTRACT

Recent rapid climate warming at the western Antarctic Peninsula (WAP) results in elevated glacial melting, enhanced sedimentary run-off, increased turbidity and impact of ice-scouring in shallow coastal areas. Discharge of mineral suspension from volcanic bedrock ablation and chronic physical disturbance is expected to influence sessile filter feeders such as the Antarctic soft shell clam *Laternula elliptica* (King and Broderip, 1832). We investigated effects of sedimentary run-off on the accumulation of trace metals, and together with physical disturbance, the cumulative effect on oxidative stress parameters in younger and older *L. elliptica* from two stations in Potter Cove (King George Island, Antarctica) which are distinctly impacted by turbidity and ice-scouring. Fe, Mn, Sr, V and Zn concentrations were slightly higher in sediments of the station receiving more sediment run-off, but not enriched in bivalves of this station. The only element that increased in bivalves experimentally exposed to sediment suspension for 28 days was Mn. Concentration of the waste accumulation biomarker lipofuscin in nervous tissue was higher in *L. elliptica* from the “exposed” compared to the “less exposed” site, whereas protein carbonyl levels in bivalve mantle tissue were higher at the less sediment impacted site. Tissue metal content and lipofuscin in nervous tissue were generally higher in older compared to younger individuals from both field stations. We conclude that elevated sediment ablation does not *per se* result in higher metal accumulation in *L. elliptica*. Instead of direct absorbance from sediment particles, metal accumulation in gills seems to indicate uptake of compounds dissolved in the water column, whereas metals in digestive gland appear to originate from enriched planktonic or detritic food. Accumulation of cellular waste products and potentially reactive metals over lifetime presumably alters *L. elliptica* physiological performance with age and may contribute to higher stress susceptibility in older animals.

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1. Introduction

The western Antarctic Peninsula (WAP) region is experiencing one of the most intense regional climate warming processes on the globe (Vaughan et al., 2003; Turner et al., 2005). Recent climate reconstructions showed that between 1957 and 2006 air temperatures increased on average by 0.11 ± 0.04 °C per decade (Steig et al., 2009). Summer mean sea surface temperatures (SST) of the

Bellingshausen Sea (Antarctic Peninsula) have been rising by more than 1 °C over the last 50 years (Meredith and King, 2005). Due to the increasing air temperatures, most of the ice sheets located in the WAP region are disintegrating (Rignot and Thomas, 2002; Cook et al., 2005; Rückamp et al., 2010) and the spatial and temporal extent of seasonal sea-ice is decreasing (Barnes and Souster, 2011). This results in increased ice impact in coastal areas, as floating ice blocks are a major product of glacier disintegration (Vaughan et al., 2003; Pritchard and Vaughan, 2007) and the intensity of ice-scouring is inversely correlated with the duration of sea-ice (Smale et al., 2008; Barnes and Souster, 2011). In addition, the intensity of melt water discharge is increasing (Vaughan, 2006; Dominguez and Eraso, 2007). These changes have marked consequences on coastal benthic communities (Barnes and Kaiser, 2007; Philipp et al., 2011). Ice-scouring can lead to changes in temporal

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and spatial zonation of coastal benthic communities (Barnes, 1999; Barnes and Conlan, 2007). Glacial melt water streams carry freshwater and eroded terrigenous material into nearshore coastal areas, resulting in freshening and shading from mineral suspension in surface waters and increasing rates of inorganic particle sedimentation (Braun et al., 2001; Dierssen et al., 2002; Monien et al., 2011). Increased amounts of suspended sediments can negatively influence the physiology of benthic, especially filter feeding, organisms (Aldridge et al., 1987; Ellis et al., 2002; Philipp et al., 2011). High concentration of inorganic particles in suspension for example increased mortality rates in New Zealand scallops (Stevens, 1987) and reduced filtration rates of bivalves such as the New Zealand horse mussel *Atrina zelandica* (Ellis et al., 2002; Safi et al., 2007), the North American zebra mussel *Dreissena polymorpha* (Madon et al., 1998) as well as the Antarctic mud clam *Laternula elliptica* (Philipp et al., 2011).

Another consequence, perhaps intuitively expected for the WAP region, may be a more intense accumulation of trace metals in filter feeding organisms. Due to its geographic isolation by the Antarctic Circumpolar current (ACC), Antarctica is still one of the most pristine environments of the world where anthropogenic metal pollution ranges at low levels (deMoreno et al., 1997; Andrade et al., 2001; Lohan et al., 2001). On the western edge of the WAP, however, run-off of eroded soil with melt-water permeating through the sediments may enhance the transport of particulate and dissolved metals into the coastal environment (Alam and Sadiq, 1993; Ahn et al., 1996, 2004). Different studies reported high concentrations of metals including Al, Cu, Fe, Zn and Mn in the region of King George Island (KGI, South Shetland Islands) compared to different localities of the WAP (Curville Island, Horse Shoe Island), or central Antarctica (Peter Ioy Island, Powell Island) (Alam and Sadiq, 1993; Ahn et al., 1996; Abele et al., 2008). The higher trace metal levels in these sediments were attributed to the local characteristics of coastal run-off fed from volcanic rock ablation as also reported for Deception Island (Antarctica), where a higher metal concentration was attributed to an active submarine volcano (Deheyn et al., 2005).

Marine bivalves ingest metal-containing inorganic particles and food material and are further able to take-up dissolved metals from porewater. The bio-available element fraction is absorbed and incorporated into the tissues and shells of the bivalves (Decho and Luoma, 1996; Langston et al., 1998; Dick et al., 2007). Many filter

feeders accumulate metal concentrations several orders of magnitude above the environmental concentrations (deMoreno et al., 1997; Langston et al., 1998). If not tightly bound to functional (enzymes) or metal storage proteins, these metals can, however, cause oxidative stress, and several other toxic effects have been described (Viarengo, 1985; Goyer, 1997; Company et al., 2008).

Laternula elliptica represents a dominant biomass component in Antarctic nearshore benthic environments (Hardy, 1972; Ahn et al., 1996; Sahade et al., 1998) and is a key species for the biodeposition of particulate organic matter at KGI (Ahn, 1997; Momo et al., 2002). The present study was aimed to link the massive glacial disintegration and sediment run-off to the trace metal content and oxidative stress status in *L. elliptica* from Potter Cove, KGI. Two populations which experience different levels of environmental disturbance (sedimentation and ice-scouring) were compared, to analyse whether high coastal run-off intensity leads to higher metal accumulation in exposed populations, but also to understand the small scale variability of metal contents and accumulation over lifetime in bivalve tissues under the present coastal run-off conditions in Antarctica.

Metal concentrations were measured in sediments and different tissues of younger and older *Laternula elliptica* specimens collected at both sampling sites, and the concentrations of lipofuscin and protein carbonyls investigated in both populations as indicators of oxidative stress levels in bivalve tissues. For better interpretation of the field data, a laboratory experiment was conducted to investigate the short-term (4 weeks) effect of high sediment pulses on the tissue specific metal concentrations and induction of oxidative stress (protein carbonyl formation) under this kind of stress exposure.

2. Material and methods

2.1. Sampling in Potter Cove

To investigate the influence of sedimentation and ice-scouring on *Laternula elliptica* in the field, subpopulations from stations L1 (Fig. 1, UTM, Z21S, WGS 84, 413557 E, 3098441 N) and L2 (UTM, Z21S, 413724 E, 3099626 N) were sampled which are under distinctly different sedimentation impacts and which also differ with respect to ice-scouring frequency (Philipp et al., 2011).

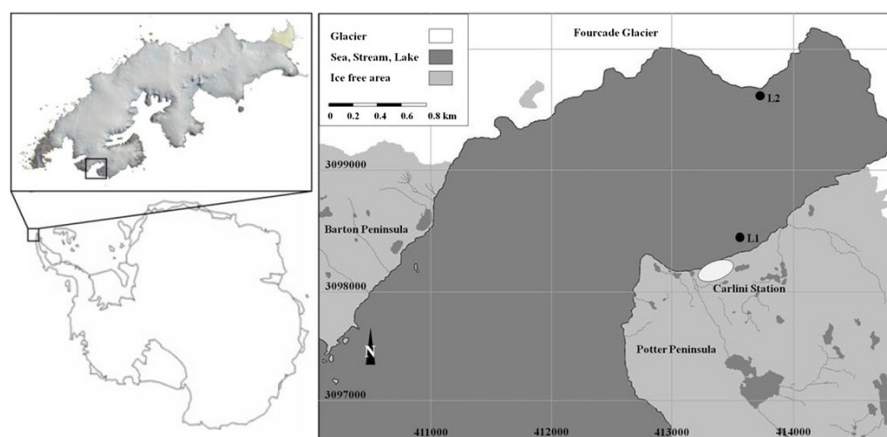


Fig. 1. Maps of the location of King George Island (KGI) on the western Antarctic Peninsula, Potter Cove located on KGI and Potter Cove. Dots mark the two sampling sites of *Laternula elliptica*. L1: “exposed” population (UTM, Z21S, 413557 E, 3098441 N), L2: “less exposed” population (UTM, Z21S, 413724 E, 3099626 N). Further the position of the Argentinean base ‘Carlini’ (former ‘Jubany’ Station) is given. Figure was drawn according to a map taken from <http://www.kgis.scar.org> (IPG, University of Freiburg; 2009).

Sediment cores for metal analysis and ratios of total organic carbon (TOC) to total nitrogen (TN) were taken in the close range of both stations using acrylic glass push corers (5 cm diameter) operated by divers (close range of L1: 5 m water depth, L2: 15 m water depth). Immediately after recovery, the cores were sampled at 1-cm intervals using a polytetrafluoroethylene extruder and a polypropylene palette-knife to avoid metal contamination. Samples were lyophilised and transported to the Institute for Chemistry and Biology of the Marine Environment (ICBM, Oldenburg, Germany) for further processing.

Smaller and larger *Laternula elliptica* individuals were hand-collected by divers in November 2006 to February 2007 in Potter Cove, King George Island (WAP, Fig. 1). Corresponding ages were inferred from shell length using a Von Bertalanffy growth model (VBGM) based on length-at-age data of the Potter Cove population taken from Philipp et al. (2008), which describes the relation between body size S_t and individual age t with: $S_t = 90.08 \times (1 - e^{-0.109 \times (t+1.665)})$.

Animals were sampled in 7 m depth at station L1 within the melt water run-off plume of the retreating Fourcade glacier. This station is more strongly exposed to sedimentation than station L2 (Philipp et al., 2011). Furthermore, at this station large floating ice blocks and brash ice entering the cove from the ocean, or calving from the large tidewater Fourcade glacier, are washed onshore by the local current system in Potter Cove (Schloss et al., 2002). Station L2 lies on the opposite coast line of Potter Cove and upstream and outside the main area of sedimentation. Neither larger ice blocks nor brash ice are frequently seen in this area, and animals were sampled at 15 m water depth. Sea surface water temperatures in the inner part of the cove, where the stations are located, range from 1.5 °C in summer down to –1.9 °C in winter. Seawater salinity ranges from 33.5 PSU to 34 PSU (Schloss et al., 2008, 2012).

Animals of both stations were kept for at least 3 days prior dissection in a constant temperature room at 1 °C in flow-through aquaria with natural seawater from the cove to empty the intestinal tract and gill tissue of ingested material which might contaminate the analysis of metal concentrations in *Laternula elliptica* tissues. Subsequently, animals were dissected and the nervous tissue fixed for histological analysis of lipofuscin (Section 2.5). Digestive gland, mantle and gills were shock frozen and stored in liquid nitrogen for the determination of protein carbonyl content and the measurement of metal concentration (Sections 2.3 and 2.6). Tissue analysis was undertaken at the Alfred Wegener Institute in Bremerhaven (Germany).

2.2. Trace metals and ratios of total organic carbon (TOC) to total nitrogen (TN) in the sedimentary environment

Quantitative XRF analysis for major and trace elements (Al_2O_3 , Fe_2O_3 , MnO, Cu, Sr, V, Zn) was carried out on ground sediment samples with a conventional wavelength dispersive X-ray fluorescence (WD-XRF) spectrometer (Philips PW 2400). Glass beads were prepared according to Schnetger et al. (2000) with slight modifications using 700 mg of the ground sample and 4200 mg di-lithium tetraborate. Beads were analysed randomly to avoid artificial trends. In order to guarantee the precision and accuracy of the XRF method, at least 7-fold measurements of several selected international reference materials (JA-2, JB-1, JG-1a) and in-house standards (PS-S, Peru-1a, Loess) were performed. The precision of the method was determined by calculating the pooled relative standard deviation after Skoog and Leary (1996). As a measure of the accuracy of the method, the relative systematic error representing the variation from the certified value was used (Skoog and Leary, 1996). Statistical evaluation is summarised in Table 1. Precision was better than 1% for the major elements (Al,

Table 1
Precision and accuracy of geochemical parameters analysed by X-ray fluorescence spectrometry.

Element/oxide	Pooled rel. SD (%)	Rel. error (%)
Al_2O_3	0.29	0.19
Fe_2O_3	0.49	–0.04
MnO	0.85	0.36
Cu	3.5	4.5
Sr	1.3	–3.9
V	2.2	0.92
Zn	1.9	–3.8

Fe, Mn) and <3.5% for the minor elements (Cu, Sr, V, Zn). Accuracy ranged from –0.04 to 0.36% (major elements) and –3.9 to 4.5% (minor elements). Al, which is known to be an immobile marker representing the lithogenic fraction, was used for normalisation in order to eliminate dilution effects by biogenic (e.g. carbonate, opal) or diagenetic (e.g. pyrite, apatite) components (Brumsack, 2006; Martinez et al., 2009).

Measurements of total carbon (TC) and total nitrogen (TN) concentrations of sediments were done by combustion on an Elementar vario EL cube CHNS elemental analyser (Hanau, Germany) equipped with a thermal conductivity detector. The content of total inorganic carbon (TIC) was determined coulometrically by a CM 5012 CO_2 coulometer coupled to a CM 5130 acidification module (UIC, Germany), whereas total organic carbon (TOC), from which TOC/TN ratios were derived, was calculated as the difference between TC and TIC.

2.3. Trace metal concentrations in different tissues of *Laternula elliptica*

Metal concentration was measured in older *Laternula elliptica* individuals from both stations (L1 and L2) of ~15 years of age (mean 76 ± 8 mm shell length). From the “exposed” station L1 additional younger animals of ~4 years were investigated (mean 43 ± 6 mm shell length). Frozen samples of gill, mantle and digestive gland tissue were lyophilised, weighed and ground in a mortar. Aliquots of 0.02–0.09 g dry weight (dwt) were individually digested in 5 mL subboiled/bidest. HNO_3 (65%, Merck) and 1 mL subboiled/distilled HF (40%, suprapur®, Merck) for 2 h at 60 °C, 3 h at 100 °C and 5 h at 160 °C in closed Teflon jars. Subsequently, teflon cups were slightly opened and samples heated in 5 mL ultrapure H_2O for 5 h at 160 °C until evaporation of the main part of acid and water. Residues of 0.5–1 mL were cooled to room temperature and volumes adjusted to 10 mL with subboiled 1 M HNO_3 . These solutions were used to determine metal concentrations (three technical replicates per sample). If necessary, the samples were diluted 1:10 to match metal calibration ranges. For metal measurement, inductively coupled plasma optical emission spectrometry (ICP-OES, IRIS Intrepid Type Duo, Thermo Nicolet GmbH) was carried out at 228.8 nm (Cd), 324.7 nm (Cu), 259.9 nm (Fe), 257.6 nm/260.5 nm (Mn, values were calculated with means of measurements with both wavelength), 407.7 nm (Sr), 292.4 nm (V) and 206.2 nm/213.8 nm (Zn, values were calculated with means of measurements with both wavelength) (Nölte, 2003). Calibrations for ICP-OES measurements were performed using multi-element solutions (Merck IV) undiluted or using dilutions ranging from 1:100 to 1:1000. The accuracy of the analytical method used for the bivalve tissues was tested using 0.2 g dwt control material (IAEA-407, North Sea Fish Tissue Homogenate; International Atomic Energy Agency, Austria; see Wyse et al. (2003) for references). Analytical results of the reference material are shown in Table 2.

Table 2

Analytical results [$\mu\text{g g}^{-1}$ dry weight] of reference material "IAEA-407" (North Sea Fish Tissues), used as quality control material for the validation of the analytical procedure (Wyse et al., 2003; <http://www.iaea.org/nael/refmaterial/iaea407.pdf>). Values as means \pm SD.

Element	Recommended value	This work [N = 9]	Percentage recovery
Cd	0.189 \pm 0.019	0.184 \pm 0.03	97
Cu	3.28 \pm 0.4	2.68 \pm 0.3	82
Fe	146 \pm 14	116 \pm 13	79
Mn	3.52 \pm 0.32	2.70 \pm 0.17	77
Sr	130 \pm 11	111 \pm 7	85
V	1.43 \pm 0.20	1.16 \pm 0.07	81
Zn	67.1 \pm 3.8	46.5 \pm 3.6	69

2.4. Sedimentation experiment at Dallmann laboratory

To investigate the direct effects of sedimentation on metal accumulation and protein carbonyl concentrations, large *Laternula elliptica* individuals (76 \pm 5 mm shell length) from the less impacted station L2 were exposed over 28 days to sedimentation. Five plastic boxes with natural sediment, each containing six *L. elliptica* individuals, were placed into either of two 180 L aquaria at ~ 1 °C seawater temperature located in a constant temperature room at 1 °C. The two aquaria were coupled with tubes, connected to the running seawater system from the cove and constantly aerated. The animals were treated over 28 days with 0 (tank 1) and 1200 mg sediment L^{-1} (tank 2). After 28 days, the animals were dissected and samples of digestive gland, gill and mantle tissue shock frozen and stored in liquid nitrogen for transportation to the Alfred Wegener Institute (Bremerhaven, Germany).

Fine grained sediment for the experiments was obtained from natural glacial run-off deposits from dried river beds in Potter Cove as described in Philipp et al. (2011). Deposits were ground in a mortar and sieved through a 50 μm mesh size sediment sieve. Sediment organic content was determined gravimetrically by combusting the sediment at 500 °C for 5 h and to determine % organic matter (OM). To achieve a constant 5% of organic matter, the algae *Desmaretia antarctica* was sampled, dried, ground to fine powder and added to the sediment to yield the final 5% organic matter. Every evening at 8 pm water flow through the aquaria was stopped and either the sediment or only the corresponding 5% organic matter (control aquaria) added. The next morning at 10 am water flow was re-started. The sediment could not be maintained in complete suspension during 14 h of exposure in the experimental tanks in spite of increased flow velocity. To record the real sediment conditions throughout the 14 h of exposure, water samples were taken every 2 h and gravimetrically analysed during one experimental run. At each sampling event, two parallel samples were taken from the different experimental units. Depending on sediment load, 100 mL – 1 L of water were filtered over pre-weighed cellulose–acetate filters (142 mm diameter, 0.2 μm pore size, Sartorius, Germany) and dried for at least 3 days at 60 °C to determine dwt. It resulted that a particulate matter concentration of 20 mg L^{-1} could be maintained in suspension for a period of more than 14 h (Fig. 2), which equalled the maximal natural sediment concentration in Potter Cove during the experimental period (Philipp et al., 2011). The set-up mimics the conditions in the field where warm days will yield a peak in glacial sediment run-off, whereas on cooler days discharge and run-off amounts decline. Water quality was checked for ammonia (Tetratest $\text{NH}_3/\text{NH}_4^+$, Tetra, Germany) and nitrite (Tetratest NO_2^- , Tetra Germany). Metal concentrations in sediments from the glacial run-off used for the sedimentation experiment were investigated in samples from three deposits from this site as described above.

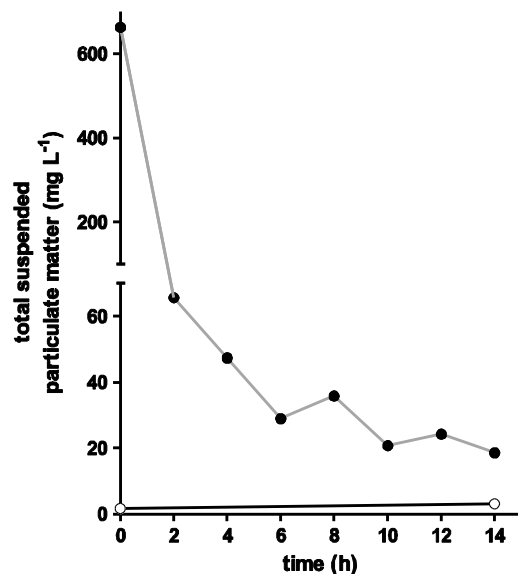


Fig. 2. Time course of total suspended particle concentration (mg L^{-1}) in the experimental tank. Water samples of the sediment treatment tank were taken every 2 h (black dots, grey line), and of the control tank at the beginning and the end of the experiment (circles, black line). Data represent mean values of two parallel samples in one tank.

Trace metal concentration of the water was assessed in water samples from a sampling station which is located north eastern of Carlini station and the laboratory from which the seawater is pumped into the aquaria (S 62°13.928', W 58°39.992'). Samples were taken between December 2010 and February 2011 in regular events over a time frame of 54 days at the surface with a bucket and at 5 m water depth with a Niskin-bottle. Samples were acidified with 2% HNO_3 and Mn, Fe and V measured in a ten-fold dilution by ICP-MS (Element 2, Thermo Scientific, Germany). In order to verify the precision and accuracy of the measurements, two international seawater reference solutions (Cass-5 and Nass-5, National Research Council Canada) were measured. Accuracy was better than 10% (Mn), 11% (Fe) and 12% (V). The precision is in the range of 4 and 36% for Mn, 3 and 25% for Fe, and 2 and 10% in case of V. In a few cases of low accuracy, a standard correction could be performed due to high precision.

2.5. Histological determination of lipofuscin in nervous tissue

Analysis of lipofuscin accumulation was undertaken in ganglia (Fig. 3) of older (~ 15 years, 75 \pm 6 mm shell length) and younger (~ 4 years, 41 \pm 6 mm shell length) *Laternula elliptica* individuals from both sampling areas. Directly after dissection, ganglia were fixed in Bakers formalin solution (100 mL formaldehyde (37%) mixed with 20 g $\text{Ca}(\text{CH}_3\text{COO})_2$ dissolved in 1 L of distilled water) over night. After fixation, the Bakers solution was removed and samples were covered with gum sucrose solution (300 g sucrose and 10 g gum arabicum in 1 L distilled water) until further processing. For embedding, samples were washed twice for 30 min in 0.2 M phosphate buffer (0.1 M NaH_2PO_4 , 0.1 M Na_2HPO_4 , pH 7.4), dehydrated in 70% (2 \times 15 min) and 100% acetone (2 \times 15 min) and transferred to 5 mL of monomer solution (80 mL 2-hydroxy-ethyl-methacrylic acid, 12 mL 2-butoxyethanol, 0.27 g benzoyl peroxide) for 12 h. Samples were embedded by mixing 5 mL of monomer solution with 100 μL of polymerisation activator solution

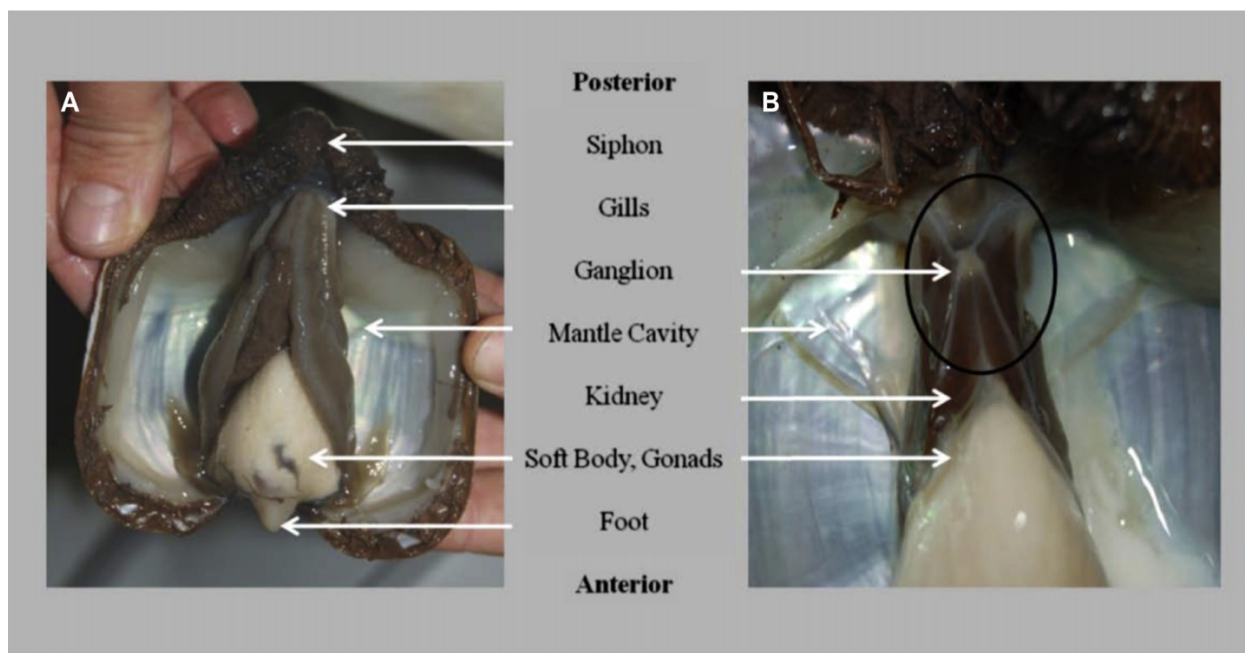


Fig. 3. *Laternula elliptica*: (A) Ventral view into the open shell. (B) Same perspective with gills removed to expose the ganglion.

(polyethylene glycol 200 and N,N-dimethylaniline, 10:1) and left to polymerise for 12 h at 4 °C. Subsequently, the samples were dried under a fume hood for another 24 h before cutting 2 μm sections with a rotation microtome (Leica, RM 2145). Sections were air dried and fixed with glycerine gelatine (Merck, Microscopy Kaisers glycerine gelatine) and a cover slide.

Samples were analysed using a Leica TCS NT confocal laser scanning microscope (instrument setting: excitation 488 nm KrAr Laser, emission 521 nm long pass filter, confocal pinhole 1, picture with 63 \times lens). Each ganglion section was scanned for auto-fluorescent granules and ten digital images (1024 \times 1024 pixels resolution) per clam/ganglion were randomly taken. Intensity of photomultiplier and offset were adjusted to maximum contrast. The percentage of tissue area occupied by lipofuscin granules was assessed using "ImageJ" software (National Institutes of Health, USA, <http://rsbweb.nih.gov/ij>) and the average % lipofuscin per area from 15 images calculated for each individual.

2.6. Protein carbonyls in mantle tissue

Protein carbonyls were measured in mantle tissue of larger animals collected from stations L1 (75 \pm 6 mm shell length, ~15 years old) and L2 (77 \pm 6 mm shell length, ~16 years old) according to Levine et al. (1990) and as described for *Laternula elliptica* in Philipp et al. (2005). The specific reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNP) results in the formation of the corresponding 2,4-dinitrophenylhydrazone, which absorbs at 360 nm (molar absorption coefficient of 22,000 $\text{M}^{-1} \text{cm}^{-1}$). Samples of mantle tissue were homogenised in HEPES buffer (50 mM) containing 125 mM KCl and protease inhibitors leupeptine, pepstatin A, phenylmethylsulfonyl fluoride, aprotinin, EDTA and MgSO_4 . Homogenates were centrifuged at 31,000 g (15 min) and 0.4 mL of supernatant mixed with 1.4 mL DNP solution (10 mM in 2 M HCl). Absorbance blanks of each sample

(HCl without DNP) were separately prepared. After incubation for 1 h in the dark at room temperature, and repeated mixing, 200 μL of trichloroacetic acid (TCA, 100%) was added and samples centrifuged at 11,000 g for 5 min. The pellet was washed 3 times with 1 mL ethanol:ethylacetate (1:1), resuspended in 600 μL guanidine hydrochloride (6 M) in 20 mM potassium phosphate (pH 2.5) and transferred to a water bath for 1 h at 37 °C. Samples were kept at 4 °C over night for complete resuspension and measured spectrophotometrically at 360 nm (Shimadzu Spectrofluorometer RF-1501), using guanidine hydrochloride as a reference. To relate the amount of protein carbonyls to the protein content, protein concentration was determined for each sample by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA Sigma A-6003) as standard.

Table 3

Metal content [$\mu\text{g g}^{-1}$ dry weight] and the corresponding ratios from Al-normalisation in sediments, sampled from cores (17 cm depth) of the "exposed" (L1), and the "less exposed" sampling station (L2).

	"Exposed" L1	"Less exposed" L2
Al	92,891 \pm 1207*	86,806 \pm 1194*
Fe	54,618 \pm 1361*	52,329 \pm 1202*
Mn	984 \pm 22*	940 \pm 18*
Cu	87 \pm 4	90 \pm 5
Sr	367 \pm 18	356 \pm 9
V	190 \pm 5*	169 \pm 5*
Zn	81 \pm 2*	76 \pm 2*
Fe/Al	0.59 \pm 0.01*	0.60 \pm 0.01*
Mn/Al [$\times 10^{-4}$]	106 \pm 3	108 \pm 1
Cu/Al [$\times 10^{-4}$]	9.4 \pm 0.5*	10*
Sr/Al [$\times 10^{-4}$]	40 \pm 2	41 \pm 1
V/Al [$\times 10^{-4}$]	20 \pm 1*	19 \pm 0*
Zn/Al [$\times 10^{-4}$]	8.7 \pm 0.2	8.8 \pm 0.16

Values as means \pm SD, n = 9–19 fractions. Mann–Whitney U-test. * = significantly different ($p < 0.05$).

2.7. Statistical analysis

Statistics were carried out using GraphPad Prism software (version 5.01). Significant differences were evaluated performing the non-parametric Mann–Whitney *U*-test.

3. Results

3.1. Field investigation

3.1.1. Metal content in sediment cores

At both stations, the upper parts of the sediment cores down to 17 cm were analysed, which corresponds to the depth profile colonised by *Laternula elliptica*. Metal concentrations did not vary significantly over the core depth sections. Therefore, means of the values measured in the different horizons were calculated for each core (Table 3). Across all analysed metals, concentrations declined in the order Fe > Mn > Sr > V > Cu > Zn. Significantly higher absolute concentrations of Fe, Mn, Sr, V and Zn were recorded at the “exposed” station L1 compared to the “less exposed” station L2 (Mann–Whitney *U*-test, $p < 0.05$). In contrast, element/aluminium ratios were significantly higher at the “less exposed” station. TOC/TN ratios from the top sections (0.5–14.5 cm depth) of the sediment cores were slightly higher at the “exposed” station (molar ratio: 9.2 ± 1.1) compared to the “less exposed” station (molar ratio: 7.3 ± 1.6). In contrast to the data obtained for metal concentrations, TOC/TN molar ratios calculated from both cores vary significantly over different depth sections (Mann–Whitney *U*-test, $p < 0.05$, Table 4). Ratios calculated for the respective 0.5 cm top layer were different and higher in the “exposed” (molar ratio 8.1) compared to the “less exposed” station (molar ratio 5.8).

3.1.2. Tissue-specific metal content in *Laternula elliptica*

Metal concentrations were highly variable among tissues (Fig. 4) and declined in the order digestive gland > gills > mantle. Particularly Cd, Cu and Sr ranged two to three orders of magnitude higher in digestive gland compared to gills and mantle. Mn did not show a conclusive pattern. Across all analysed metals, Fe was present in the highest concentrations, whereas Cr, Pb, Co and Ni were below detection limit of the method. Metal concentrations declined in the order Fe >> Zn > Cu, Sr > Cd, Mn > V in all tissues and in both age classes.

Comparing older individuals from L1 and L2, significantly higher levels of Cd, Fe and Mn in digestive glands, Cd and Cu in gills and Cd, Cu, Fe, Mn, Sr, V and Zn in mantle tissue were recorded at the “less exposed” station L2 compared to the “exposed” station L1. In no tissue, trace metal content was higher in animals from L1 compared to L2 individuals.

Tissue-specific metal concentrations further varied between size/age-groups (recorded only for station L1) with more

pronounced age-dependent differences in digestive gland and gill compared to mantle tissue. Cd, Fe, Sr and V were more highly concentrated in digestive gland and gill tissues of older (L) than younger (S) individuals ($L > S$). Only Cu (gill), Mn (digestive gland) and Zn values (gill and digestive gland) were higher in tissues of the younger compared to the older individuals ($L < S$). Thus, overall metal concentrations were higher in older compared to younger individuals.

3.1.3. Markers of oxidative stress

Roundish to irregular shaped lipofuscin granules were abundant in the cytoplasm of *Laternula elliptica* ganglia cells, where they usually formed clusters (Fig. 5). Concentrations ranged from <0.05 to 6.6% lipofuscin per area unit. Granular density was significantly ($p < 0.0001$) higher in ganglia of older than of younger animals at both stations (Figs. 5 and 6). Lipofuscin density values were moreover significantly higher in older animals from the “more exposed” L1 than from the “less exposed” L2 station ($p < 0.05$). Differences between younger animals from both stations were insignificant (Fig. 6).

Protein carbonyl values were measured in mantle tissue of older *Laternula elliptica* from both stations. The results contrast the pattern observed for lipofuscin accumulation in nervous tissue. Mean mantle protein carbonyl concentrations were significantly higher ($p < 0.05$) in animals from the “less exposed” (L2: $2.12 \text{ nmol mg}^{-1}$ total protein $\pm 0.83 \text{ SD}$, $N = 19$) compared to the “exposed” site (L1: $1.60 \text{ nmol mg}^{-1}$ total protein $\pm 0.56 \text{ SD}$, $N = 18$).

3.2. Laboratory experiment

Animals were exposed 14 h/day for 28 days to either 0 or 1200 mg sediment which contained metal concentrations of 62,640 (Fe), 1026 (Mn), 116 (Cu), 386 (Sr), 224 (V) and 83 (Zn) $\mu\text{g g}^{-1}$ dwt (measured in three deposits of the sediment collection area). In comparison, seawater in Potter Cove contains only mean values of $4 \mu\text{g L}^{-1}$ Fe, $1.2 \mu\text{g L}^{-1}$ Mn and $1.7 \mu\text{g L}^{-1}$ V in the first 5 m of the water column (Supplementary Table S1). Cu, Cd and Zn in seawater were below the detection limit.

Only Mn concentrations increased significantly in digestive glands of sediment exposed compared to control animals (Fig. 7). Concentrations of Cd, Fe, Zn, Cu and Sr remained constant over the exposure time. Cd and Sr were higher in digestive gland than in gills, as also seen in the measurements of the field animals. Protein carbonyl content in gills of *Laternula elliptica* did not change in the high sedimentation treatment with $10.53 \text{ nmol mg}^{-1}$ total protein $\pm 0.82 \text{ SEM}$ in control animals and $10.62 \text{ nmol mg}^{-1}$ total protein $\pm 1.14 \text{ SEM}$ in sediment treated individuals (controls: $N = 8$, sediment treated: $N = 9$).

4. Discussion

4.1. Does enhanced sedimentation increase metal concentration in *Laternula elliptica* tissues?

Higher total concentrations of several metals in sediments at L1, but higher ratios of Al-normalised metals in the sedimentary environment of the “less exposed” L2 station, suggest different geochemical reservoirs at both stations. The differences, however, are subtle and may reflect measuring artefacts as much as they may reflect different metal mobilisation and real differences of source material at both stations. The bioavailability of sedimentary metals depends on numerous physicochemical or (micro-) biological properties, such as the presence of organic matter and sulphide concentrations, as well as the mineral content of the sediments (John and Leventhal, 1995). The differences we found between the

Table 4
Molar TOC/TN ratios obtained from top sections of sediment cores, sampled from the “exposed” (L1), and the “less exposed” sampling station (L2).

Sampling depth (cm)	“Exposed” L1 TOC/TN (molar)	“Less exposed” L2 TOC/TN (molar)
0.5	8.1	5.8
2.5	8.5	7.2
4.5	8.6	8.2
6.5	9.0	8.0
8.5	8.7	8.0
10.5	11.6	9.1
12.5	9.8	7.8
14.5	9.1	4.1
Average \pm SD	9.2 ± 1.1	7.3 ± 1.6

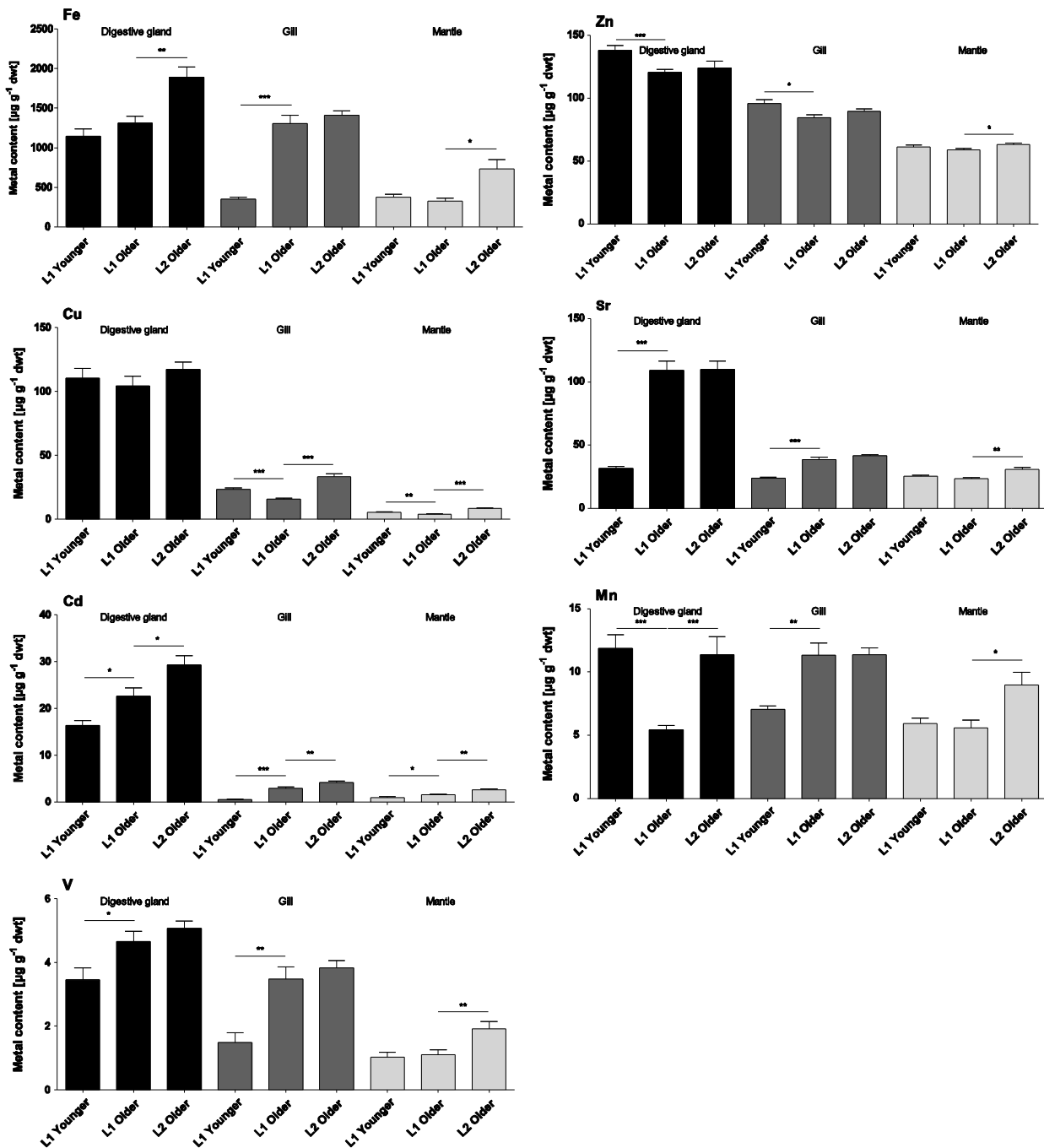


Fig. 4. Metal content [$\mu\text{g g}^{-1}$ dry weight (dwt)] in digestive gland, gill and mantle tissue of older *Laternula elliptica*, sampled from the “exposed” (L1, mean shell length 74 ± 7 mm, ~ 14 years, $N = 16\text{--}18$ animals), and the “less exposed” sampling station (L2, mean shell length 76 ± 8 mm, ~ 15 years, $N = 18\text{--}28$ animals) as well as additional younger individuals from the “exposed” station L1 (mean shell length 43 ± 6 mm, ~ 4 years, $N = 10\text{--}12$ animals). Values are shown as means \pm SEM. *, ** or *** = significantly different ($p < 0.05$, $p < 0.01$ or $p < 0.0001$).

cores taken at both stations do not appear sufficiently large to explain the differences in animal tissues from both stations. Although higher metal concentrations in the bivalves at the “less exposed” L2 station seem consistent with higher Al-normalised metal values at that station, this does not necessarily reflect

uptake of metal containing sediments into the animals’ digestive system.

Instead, it may indicate metal uptake is via ingestion of metal enriched suspended organic particles from the sediment surface or water column. Animals at station L2 which are less impacted by

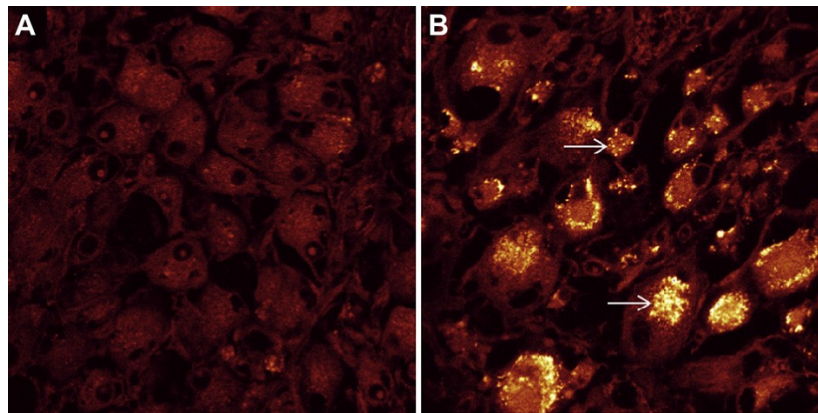


Fig. 5. Fluorescent lipofuscin granules (white arrows) in ganglia of *Laternula elliptica*. Shown are representative sections from younger (A: 41 ± 6 mm, ~4 years) and older (B: 75 ± 6 mm, ~15 years) individuals. Excitation = 488 nm, emission ≥ 521 nm, digital confocal laser scanning microscopy ($\times 63$).

mineral suspension may ingest proportionally more digestible food particles (phytoplankton and detrital material) that enrich heavy metal content in different bivalve tissues (see below). In agreement with this, lower TOC/TN ratios were detected in the top sediment layer at the “less exposed” station L2, which points towards a higher amount of freshly deposited organic matter at this site.

4.2. Does the Potter Cove environment impose higher metal total concentrations on its inhabitants than other regions of the WAP?

The order of metal concentrations in *Laternula elliptica* digestive glands and gills is similar to those detected in *L. elliptica* from other

areas around KGI (Ahn et al., 1996, 2001; Curtosi et al., 2010) and also from the more southern WAP station Rothera (Lohan et al., 2001). Total metal concentrations in digestive gland tissue range around: 1068–2003 $\mu\text{g g}^{-1}$ tissue dwt Fe, 90–153 $\mu\text{g g}^{-1}$ tissue dwt Zn, 38–75 $\mu\text{g g}^{-1}$ tissue dwt Cu, 4–19 $\mu\text{g g}^{-1}$ tissue dwt Mn and 11.5–19 $\mu\text{g g}^{-1}$ tissue dwt Cd; and in gill tissue around: 1260–1998 $\mu\text{g g}^{-1}$ tissue dwt Fe, 124–206 $\mu\text{g g}^{-1}$ tissue dwt Zn, 8–21 $\mu\text{g g}^{-1}$ tissue dwt Cu, and 15–45 $\mu\text{g g}^{-1}$ tissue dwt Mn, 3–7 $\mu\text{g g}^{-1}$ tissue dwt from *L. elliptica* individuals of KGI Maxwell Bay (Collins Harbour, Marian Cove and Potter Cove; Ahn et al. (1996)), which fit our data for the respective tissues. KGI individuals, however, accumulate at least twice as much Fe compared to Rothera animals (Lohan et al., 2001). With the exception of Cu, all other metals are less concentrated in *L. elliptica* tissues at Rothera compared to KGI. In respect to Cu, the authors hypothesise that these high concentrations may be due to aircraft landing and dump material from the 1970s and early 1980s near the bivalve habitat at Rothera (Lohan et al., 2001).

Several fold higher Fe concentrations in animals from Maxwell Bay than at the Rothera station may point towards differing bio-availabilities of Fe and, generally, of the geochemical characteristics in both environments. This may relate to the enhanced coastal run-off at KGI in the rapidly warming northern WAP region compared to the Rothera region where air temperatures are still a few degrees cooler (Ducklow et al., 2007).

Higher bioavailability may result from the volcanic nature of the KGI bedrock. Deheyn et al. (2005) studied metal concentrations in sediments and animals of Deception Island, an active submarine volcano in the Antarctic Peninsula region and compared these with results obtained from King George Island as a non-active volcanic Island. Metal composition in the sediment and different benthic organisms of Deception Island indicate that trace elements of volcanic origin are available to benthic filter and deposit feeders (Deheyn et al., 2005). This may also apply to the Potter Cove environment. Fe is one of the most abundant metals in seafloor sediment samples and soft tissues of benthic organisms in Potter Cove and in Deception Island.

Short-term experimental sediment exposure did not increase metal contents in bivalve tissues. Only Mn was elevated in gills (but not in digestive gland) of bivalves treated with high sediment loads for 28 days. The higher Mn levels found in gills of sediment-exposed animals may indicate that sediment particles might have been accumulating at the gill surface, and perhaps at times might have clogged the gills of the exposed bivalves. As Mn

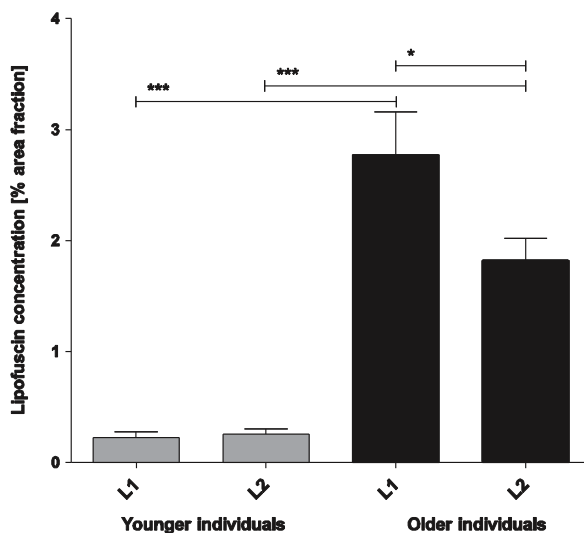


Fig. 6. Lipofuscin content [% area fraction] in ganglia sections of older *Laternula elliptica* individuals, sampled from the “exposed” (L1, mean shell length 75 ± 6 mm, ~15 years), and the “less exposed” sampling station (L2, mean shell length 75 ± 7 mm, ~15 years) as well as younger individuals from the “exposed” station L1 (mean shell length 40 ± 8 mm, ~4 years) and the “less exposed” station L2 (mean shell length 41 ± 3 mm, ~4 years). Values are shown as means (\pm SEM), younger individuals: $N = 7$ (L1) and 9 (L2), older individuals: $N = 17$ (L1) and 26 (L2). * or *** = significant difference between groups ($p < 0.05$ or $p < 0.0001$).

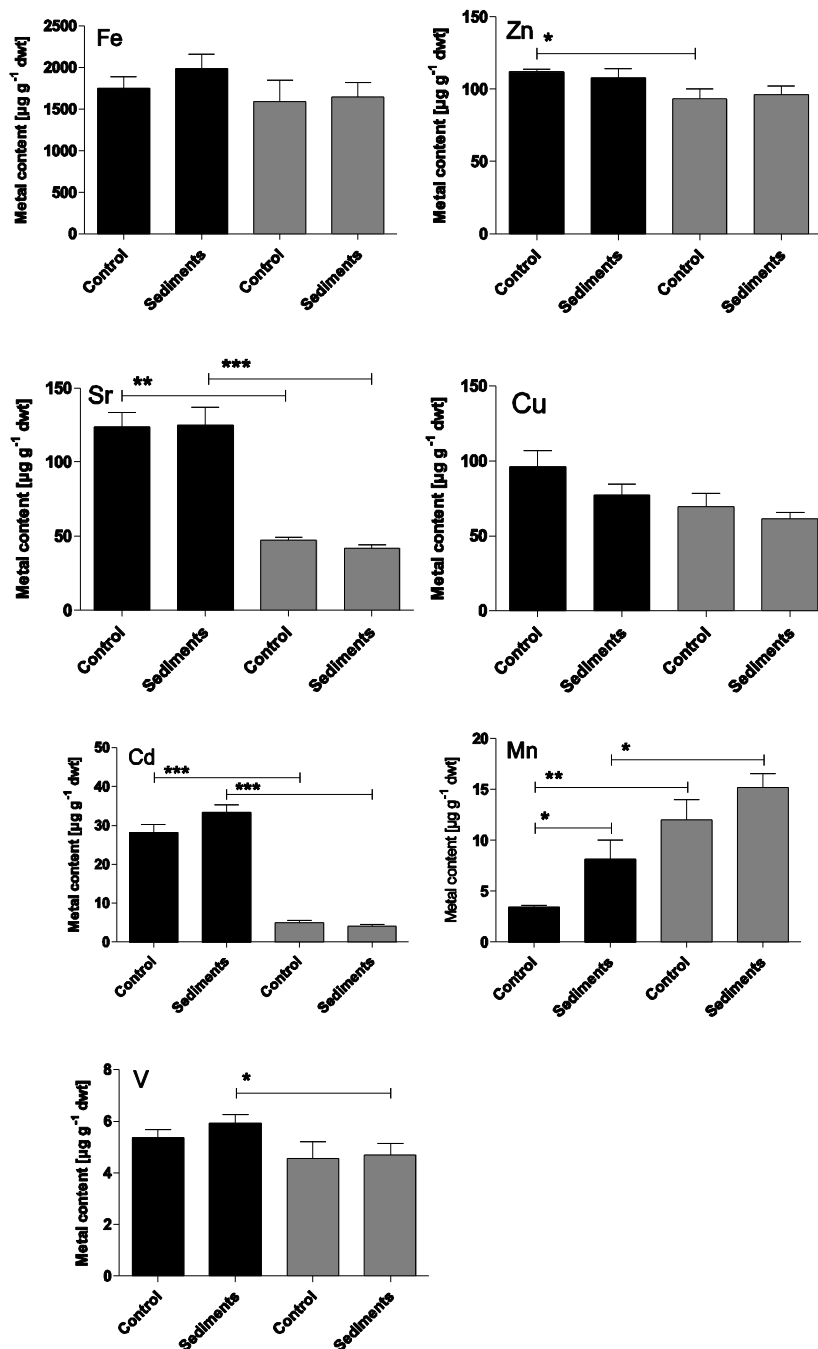


Fig. 7. Metal content [$\mu\text{g g}^{-1}$ dry weight (dwt)] in digestive gland (black columns) and gill (grey columns) tissue of *Laternula elliptica* after 28 days of experimental sediment treatment (1200 mg L^{-1} , 12 h per day) or control conditions without sediment. Size range = 76 ± 5 mm shell length, ~15 years, $N = 8-9$. Values are shown as means \pm SEM, * = significantly different ($p < 0.05$).

concentrations in the marine habitat or the sediment to which the animals were exposed are nearly a hundredfold higher compared to concentrations in *Laternula elliptica* tissues, accumulation of Mn might be easier to detect compared to the other investigated metals. This indicates Mn to be a good marker for flocculating sediment particles attached to tissue surfaces such as gills. In contrast, Mn was never enriched in the digestive gland of

sediment exposed animals and neither were the other metals. Thus, 28 days of exposure were probably too short to increase metal concentrations in tissues of exposed animals. Furthermore, the low *in situ* Mn levels in bivalve tissues compared to surface sediments suggests *L. elliptica* efficiently sorts edible and inorganic particles and excludes sediment particles before ingesting filtered material.

Cu and Cd were particularly elevated in the digestive gland compared to the other tissues, which is consistent with the data of Curtosi et al. (2010) who measured metal concentrations in digestive gland, gonad and muscle tissue of Potter Cove *Laternula elliptica*, and also Bargagli et al. (1996) who worked with *L. elliptica* from Terra Nova Bay. Antarctic diatoms are particularly rich in Cd (Bargagli et al., 1996) and also benthic macroalgae accumulate Zn, Cu and Cd from seawater (deMoreno et al., 1997). Benthic diatoms resuspended by wind and wave action in shallow water represent an important food source for *L. elliptica* (Ahn, 1994), and also the consumption of macroalgal detritus may be a source of metal uptake in *L. elliptica*. Metal accumulation was generally higher in older compared to younger *L. elliptica*, indicating irreversible accumulation over lifetime. In line with Ahn et al. (2001), Mn, Zn and Cu were, however, higher in digestive glands of the younger compared to the older individuals. Ahn et al. (2001) attributed this to the higher feeding activity of small, young individuals (Ahn, 1993). Mn concentrations, in particular, were markedly higher in digestive gland tissues of younger compared to older individuals which may also indicate inefficient sediment sorting in young animals at the more impacted station. We have shown recently that large bivalves from L1 compared to small L1 individuals reduce respiration and thus ventilation under high sedimentation impact. This behaviour may help older bivalves of this station in keeping metal pollution in body tissues low (Philipp et al., 2011).

4.3. Trace metal accumulation, protein carbonyls and lipofuscin enrichment: what stress markers stand for in *Laternula elliptica*

Protein carbonyls are formed through oxidation of cellular proteins and are sensitive markers of oxidative stress. Loss of enzyme activity and protein function are counted under the consequences of oxidative protein modification (for review see Berlett and Stadtman, 1997). Significantly higher concentrations of Cu and Fe in mantle tissue of older bivalves collected at station L2 could partly be a cause for the increased protein oxidation in bivalve mantle tissue from this station. Both ions are powerful catalysts of oxidative damage through Fenton-type hydroxyl generating reactions (Valko et al., 2005; Prousek, 2007), and our study indicates that there may indeed be a correlation between tissue metal load and protein oxidation in the bivalves. This agrees with Sabatini et al. (2011), who found protein carbonyl groups to increase in the bivalve *Diplodon chilensis* after several weeks of dietary copper exposure. Protein carbonyls are, however, very general indicators of oxidative stress, caused by many stressors such as hyperthermia or starvation (Ansaldo et al., 2007; Park et al., 2008) and future studies may find additional causes for the observed differences protein carbonyl concentrations between L1 and L2 animals.

Whereas the differences in protein carbonyl concentrations between both stations agree with tissue metal contents, lipofuscin accumulation in bivalve nervous tissue seems to rather reflect the overall environmental stress scenarios at both stations. Lipofuscin is an intra-lysosomal, non-degradable substance composed of cross-linked protein and lipid residues. It is regarded as a final product of stress induced oxidative damage but also accumulates over age in *Laternula elliptica* and other bivalve tissues (for review see Philipp and Abele, 2010). These granular deposits which are thought to disturb intracellular fluid transport and particle flux, accumulate preferentially in postmitotic nervous tissues (Brunk and Terman, 2002; Terman and Brunk, 2004).

Concentrations of lipofuscin and lipofuscin-like aggregates were shown to be elevated after metal exposure in several bivalves such as *Sunetta scripta*, *Perna viridis*, *Mytilus galloprovincialis*, *Anadara cf. inaequivalvis* or *Chamelea gallina* (Regoli, 1992; Mathew and

Damodaran, 1997; Kolyuchkina and Ismailov, 2011). In our study, lipofuscin accumulation in larger *Laternula elliptica* was higher at the “exposed” L1 station with higher sediment loads and iceberg scour frequencies than at L2. The concerted impact of different stressors at L1 may thus explain more rapid accumulation of the oxidative waste product in nervous tissue of bivalves.

Optical analysis of post-mitotic nervous tissue by fluorescence microscopy demonstrated lipofuscin granules in bivalve ganglia cells to be mainly homogeneously dispersed in the cytoplasm and sometimes also concentrated in larger assemblies around the nucleus. Autofluorescent granules were of similar shape as lipofuscin granules detected in nervous tissues of other marine invertebrates such as brain sections of polar crustaceans (Bluhm et al., 2001) and ganglia sections of European lobster (Sheehy et al., 1999). Whereas extractable lipofuscin was shown to accumulate in mantle tissue of Potter Cove *Laternula elliptica* over age with a 2-fold increase between younger (~3 years) to older (~15 years) individuals (Philipp et al., 2005), in the present study of nervous tissue, the change within this age range was almost 12-fold, indicating a much stronger signal of age pigment accumulation in nerve cells.

The opposed results obtained for both markers between the two stations may result from the fact that oxidised proteins arising from oxidative stress are rapidly metabolised in proliferating tissues such as the mantle (Strahl and Abele, 2010). Thus, the carbonyl measurements may rather reflect a momentary steady state level of oxidative damage, whereas lipofuscin granule accumulation in nerve cells can be considered a biomarker for life-long stress impact. Furthermore, in the present study carbonyl content was measured exclusively in the soluble protein fraction. In a recent investigation of oxidative stress related protein oxidation, large differences in carbonyl content between the soluble and insoluble protein fraction were noted (Bhattacharya et al., 2011). The insoluble fraction was found to be more suitable for measurements of oxidative stress-related protein damage. Oxidised proteins can initiate cross-linking of proteins, which makes them insoluble and resistant to protease digestion (Hallén, 2002). This fraction may therefore better reflect long-term accumulation of protein oxidation than the soluble fraction measured in the present study. The results indicate that monitoring studies comparing distinctly and chronically impacted sites should focus on post-mitotic tissues and on non-degradable markers, such as lipofuscin.

Older *Laternula elliptica* individuals were previously found to be more susceptible to environmental stressors than younger individuals, and Philipp et al. (2011) therefore suggested that increasing environmental stress levels around the western Antarctic Peninsula will lead to changes in *L. elliptica* population structure. Higher accumulation of metals and oxidative damaged molecules in older individuals may further render them more vulnerable to stress scenarios and corroborate this assumption. Comparative studies of habitat-specific *L. elliptica* population structures and specific physiological stress markers in animals from the southern WAP region (such as Rothera) may indicate whether the combined stress scenario of warming coastal waters and increased sediment run-off at King George Island compared to the colder South already impacts *L. elliptica* populations at the northern WAP under the present warming trends.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ecss.2012.06.003>.

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MANUSCRIPT III**Age dependent expression of stress and antimicrobial genes in the hemocytes and siphon tissue of the Antarctic bivalve *Laternula elliptica* exposed to injury and starvation**

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Abstract

Increasing temperatures and glacier-melting at the Western Antarctic Peninsula (WAP) are causing rapid changes in shallow coastal and shelf systems. Climate change related rising water temperatures, enhanced ice scouring as well as coastal sediment run-off in combination with changing feeding conditions and microbial community composition will affect all elements of the nearshore benthic ecosystem, a major component of which is the Antarctic soft shell clam *Laternula elliptica*. 454 RNA sequencing was carried out on tissues and hemocytes of *L. elliptica*, resulting in 42,525 contigs of which 48% were assigned putative functions. Changes in the expression of putative stress response genes were then investigated in hemocytes and siphon tissue of young and old animals subjected to starvation- and injury-experiments in order to investigate their response to sedimentation (food dilution, starvation) and iceberg scouring (injury). Analysis of antioxidant defense (*Le*-SOD, *Le*-catalase), wound repair (*Le*-TIMP, *Le*-chitinase), stress and immune response genes (*Le*-HSP70, *Le*-actin, *Le*-theromacin) revealed that most transcripts were more clearly affected by injury rather than starvation. The up-regulation of these genes was particularly high in the hemocytes of young, fed individuals after acute injury. Only minor changes in expression were detected in young starved and old animals. The stress response of *L. elliptica* thus depends on the nature of the environmental cue and on age. This has consequences for future population predictions as the environmental changes at the WAP will differentially impact *L. elliptica* age classes and is bound to alter population structure.

Keywords

Western Antarctic Peninsula, transcriptome, climate change, antioxidants, immune response

Introduction

The molecular immune repertoire and stress response of marine and fresh water bivalves is still poorly understood (summarized recently by Tomanek (2011), Venier et al. (2011) and Philipp et al. (2012b)). Bivalves rely exclusively on the innate immune system (Bachere et al. 1995; Canesi et al. 2002). Hence, when the physical barriers of shell and epithelia have been breached as a result of injury and/or infection, invading microorganisms, non-self particles and cell debris are recognised and eliminated by humoral and cellular mechanisms. Humoral responses comprise the permanent or inducible biosynthesis of antimicrobial peptides or other defensive peptides (Bettencourt et al. 2007). Cellular reactions include phagocytosis and the generation of reactive oxygen species (“oxidative burst”). These are mediated by the hemocytes (Donaghy et al. 2009; Husmann et al. 2011; Pruzzo et al. 2005). These cells have key functions not only in immunity, but also the maintenance of physiological homeostasis. They are involved in the inflammatory response, wound repair, shell formation, transport of nutrients, digestion, as well as excretion of biological active molecules such as enzymes or antimicrobial peptides (Canesi et al. 2002; Donaghy et al. 2009; Kadar 2008). In order to regenerate integrity and function in organs after injury, hemocytes migrate towards the wound and infiltrate the affected tissue (de Eguileor et al. 1999; Ottaviani et al. 2010). A granulated tissue is produced, which seals the wound with the synthesis of extracellular matrix components and, finally, re-epithelialisation can occur (Ottaviani et al. 2010). The ability to mount such cellular defence reactions in order to maintain organism homeostasis under environmental disturbance is crucial for survival (Hawley and Altizer 2011; Mydlarz et al. 2006). However, it is important to note that the immunocompetence of bivalves is related to general ecological factors such as temperature, salinity and food availability (Fisher et al. 1987; Hawley and Altizer 2011; Butt et al. 2007; Hegaret et al. 2004). It is also affected by artificially induced stressors such as xenobiotics and pollution (Oliver et al. 2001; Chu et al. 2002) or injury and mechanical disturbance (Husmann et al. 2011; Ballarin et al. 2003). Indeed, the latter was found to promote viral, bacterial and protozoan diseases (Morley 2010) as well as to cause extensive mass mortalities upon bacterial infection (Beaz-Hidalgo et al. 2010; Hawley and Altizer 2011).

The effects of environmental stress on the cellular immunity of bivalves have been largely investigated in terms of hemocyte abundance or phagocytic and enzymatic activity. For example, differential food quality was found to influence phagocytotic activities, reactive oxygen species production and abundance of free floating hemocytes in *Crassostrea gigas* and *Ruditapes philipinarum* (Delaporte et al. 2006; Delaporte et al. 2003). In large sized

Laternula elliptica, reduced hemocyte numbers were detected in animals subjected to starvation treatments compared to constant feeding (Husmann et al. 2011). Further, in the clam *R. philippinarum* (Oubella et al. 1993) and the Sydney rock oyster (*Saccostrea glomerata*), starvation reduced hemocyte numbers and phenoloxidase, peroxidase and acid phosphatase activities decreased (Butt et al. 2007). Similarly, mechanical disturbance (shaking) reduced hemocyte abundance and oxidative burst activity in abalones *Haliotis tuberculata* and blue mussels, *Mytilus edulis* (Bussell et al. 2008; Malham et al. 2003). In contrast, increased hemocyte numbers were observed in response to shell injury in deep-sea vent mussels, *Bathymodiolus azoricus* (Kadar 2008) and in the Antarctic clam *L. elliptica* (Husmann et al. 2011). This difference in response may be due to the fact that shell injury causes a major breach in animal integrity, whilst starvation may produce energetic trade-offs in animal biochemistry.

A recently investigated aspect of the bivalve defense response is the regulation of molecular effectors such as functional peptides and proteins in hemocytes and soft body tissues (Koutsogiannaki and Kaloyianni 2010; Tomanek 2011). Other proteomic/transcriptomic studies in bivalves point towards a common set of stress-induced proteins (Tomanek 2011). These comprise heat shock proteins (HSPs) involved in the stabilisation of proteins (Clark et al. 2008a; Santoro 2000), as well as molecules participating in oxidative stress regulation (Canesi et al. 2010; Monari et al. 2008; Park et al. 2009), tissue damage repair (De Decker and Saulnier 2011; Montagnani et al. 2001), tissue development (Badariotti et al. 2006; Badariotti et al. 2007b; Tirape et al. 2007) or antimicrobial defence (Xu et al. 2010).

At the Western Antarctic Peninsula (WAP) region, recent rapid aerial warming has caused profound environmental changes, including warming of the water in shallow coastal and shelf areas and rapid glacier disintegration (Turner et al. 2009; Cook et al. 2005; Schloss et al. 2012). Glacier melt water streams carry high amounts of terrestrial mineral suspensions into the marine coastal environment, and therefore higher levels of glacier melt results in increased nearshore marine sedimentation loads (Dominguez and Eraso 2007; Schloss et al. 2012). The calving of glacial fronts and ice-shelves produces increases in the amounts of floating brash ice and icebergs and hence more ice-scouring in shallow coastal areas (Turner et al. 2009; Barnes and Souster 2011; Brown et al. 2004). Such changes will have marked consequences for benthic animals colonising coastal areas around the WAP (Barnes and Conlan 2007; Barnes and Kaiser 2007).

To investigate the implications of these changes on, and also predict future responses of the nearshore marine benthic ecosystem, this study investigated the effect of high sediment

concentration and mechanical injury on a major component of the Antarctic benthic ecosystem, the filter feeding bivalve *Laternula elliptica*. This long lived (36 years) soft shell clam is endemic to the maritime Antarctic and one of the most abundant sentinel species of the infauna in soft bottom habitats (Philipp et al 2005a, Philipp et al 2011). Due to its circumpolar distribution and contribution to the benthic-pelagic coupling it plays a key role within the Antarctic coastal ecosystem (Ahn 1997; Momo et al. 2002; Philipp et al. 2011).

The aim of these experiments was to address the potential effects of altered food supply (by enhanced sedimentation and dilution of planktonic food with mineral suspension), shell cracks and tissue-injury by scouring ice blocks on a keystone species.

Previous data on this species revealed a higher susceptibility to environmental stress in older age classes of *L. elliptica*. Older *L. elliptica* exhibited reduced metabolic rates during exposure to high sediment loads and featured lower survival rates after injury compared to younger cohorts (Philipp et al. 2011). Furthermore, they are reported to be more sensitive to increased water temperatures (Peck et al 2007) and have a more limited ability to re-burrowing into the sediment when unearthed by icebergs (Morley et al. 2007; Peck et al. 2004; Philipp et al. 2011). They also exhibit lower oxidative defense capacities and higher levels of oxidative damage (Philipp et al. 2005a), and their hemocytes are less able to mount an oxidative burst response compared to hemocytes of younger specimens (Husmann et al. 2011).

If this differing physiological fitness and increased sensitivity to environmental challenge found in older animals leads to selective mortality in the corresponding age-classes, it is expected that the age and size structure of *L. elliptica* populations will change in the near future. This study investigated the changes in gene expression levels in response to injury and starvation in younger and older individuals of *L. elliptica* to identify age specific responses. 454 high-throughput sequencing (GS FLX, Roche-454 Life Sciences) was used to generate an extensive RNA sequence database to enable a more comprehensive choice of candidate genes for in-depth investigations. The expression changes of selected candidate genes involved in the general stress- and immune response were studied in the hemocytes and siphon tissue of young and old *L. elliptica* individuals from starvation and injury experiments. The potential impact of these factors on future population structures is discussed.

Material and Methods

Sampling of animals

Laternula elliptica individuals were collected by divers in Potter Cove, King George Island, Antarctic Peninsula (62°14'S, 58°40'W), between November 2008 and February 2009 at 7-15 m depth. Animals were classified as “large” with 7.2 ± 0.5 cm (mean, SD) and “small” with 4.8 ± 0.5 cm shell length. Approximate ages of the animals were estimated from shell size using a *L. elliptica* von Bertalanffy growth model from Potter Cove (Philipp et al. 2008). Ages within the group of “small” individuals ranged between 4 to 6 years, and in the “large” group > 11 years. At the Dallmann Laboratory on the Argentinean Station Carlini (formerly named Jubany), animals were kept in four 180 L tanks (40 small and 40 large animals per tank) for 10 days in aerated, natural seawater from Potter Cove at a constant temperature of 1°C without sediment. Every second day 50% of the water in the holding system was renewed to ensure good water quality. Following this acclimation period, individuals from both size/age groups were exposed to different stress treatments.

Stress experiments

Small and large *L. elliptica* were subjected to starvation and injury. Half of the animals from each size group were starved by keeping them in filtered seawater of 0.5 μm final pore size (treatment “starvation”). The other half of the group was kept as “fed controls” in unfiltered seawater which was additionally enriched with dissolved and particulate nutrients (treatment “food”). The mixed diet consisted in live microalgae as well as “artificial detritus” i.e. freeze dried and ground macroalgae (*Ascoseira mirabilis*) from Potter Cove, as well as krill and red bloodworms obtained from Tetra (Tetra Delica; Melle, Germany). Microalgae were collected with fine nets in Potter Cove and cultured in 2L-bottles at 1°C. Animals were fed every second day.

After 21 days 50% of the small and large animals from the starvation and food treatment were artificially injured. 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 infliction, the animals were allowed to recover in their tank of origin under the respective food condition. Two days after the injury event i.e. at day 23 after starting the food treatments, hemocyte cells and tissues were sampled for gene expression analysis from all groups. Additional samples were taken at day 44 after the initial start of the different food treatments, i.e. in case of the injured animals after 21 days of regeneration from

the injury. Hemolymph fluid containing the hemocyte cells was individually collected by inserting a 16-gauge needle with a 10 ml-syringe into the posterior adductor muscle and slowly withdrawing the hemolymphatic fluid. Two aliquots of 1.5 ml each were sampled and immediately centrifuged 10 min at 1000 g and 2°C. The supernatant was discarded and the hemocyte pellet flash-frozen in liquid nitrogen. Subsequently animals were sacrificed and the different tissues flash-frozen in cryovials in liquid nitrogen. The hepatosomatic index (HSI) was calculated from digestive gland fresh weight and total tissue fresh weight to provide indications on the physiological state of the experimental animals as:

$$\text{HSI} = (\text{fresh weight of digestive gland} / \text{total tissue fresh weight}) * 100$$

Frozen samples were transported to the Institute of Clinical Molecular Biology in Kiel, where they were kept at -80°C until further processing.

***L. elliptica* RNA sequence database generation**

In order to maximize the number of expressed transcripts and facilitate the identification of candidate genes involved in injury, immune system and general stress response processes, samples from both *L. elliptica* age groups and the different treatments were used for RNA sequence database generation. Total RNA was extracted separately from samples of digestive gland, gill and siphon (one small and one large animal each from the treatment “starvation/acute injury” and “food/acute injury”) as well as from hemocyte cells (4 small animals from treatment “starvation”, 5 small from “food”, 4 large from “starvation/acute injury” and 4 small as well as 4 large from “food/acute injury”). Tissues were ground in liquid nitrogen and the total RNA of hemocyte cells and tissues extracted using the Qiagen RNeasy kit in combination with Qiagen QiaShredder columns (Qiagen, Hilden, Germany), including DNase digestion steps. According to the manufacturer’s instructions the quality of the extracted RNA was checked photometrically by a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) and only samples showing 260/280 nm absorbance ratios of >2.2 were further processed. Equal amounts of total RNA were combined into to a single pool and mRNA isolated using the Oligotex mRNA purification kit (Qiagen, Hilden, Germany). Two cDNA samples were generated from the extracted mRNA by using two alternative ways: (1) by the BD SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Palo Alto, CA, USA) and (2) by the GS FLX Titanium Series cDNA rapid library Preparation Method Protocol (October 2009, Rev. Jan 2010, Roche).

Pyrosequencing, assembly and processing of 454 sequences

cDNA was sequenced on the Genome Sequencer FLX system (454 Life Sciences, Branford, CT, USA) using Titanium chemistry according to the manufacturers' protocol at the Institute of Clinical Molecular Biology (ICMB). The resulting 454 sequences were extracted from FLX output files using the 'sffinfo' script from Roche. Prior to assembly, polyA tails, SMART primer sequences, 454 adapter sequences and sequences of bad quality (quality score <11) were removed from the data set using 'seqclean' and 'cln2qual' software (TGI - The Gene Index Project). Read sequences < 40bp after the filtering were also discarded. 489,924 of the initial 500,954 sequences remained and were assembled using the 'GS De novo Assembler 2.3' software (NEWBLER, Roche/454 Life Sciences). Assembly parameters were: minimum overlap length = 40, minimum overlap identity = 90. The resulting contigs and singletons were further repeatedly assembled using TGICL (Cap3) software and the following filter parameters: minimum overlap length 40-360 bp, minimum overlap identity 90-100%. For a last contig refinement the initial filtered reads were mapped against the assembled contigs using AMOScmp software (<http://sourceforge.net/apps/mediawiki/amos/index.php?title=AMOScmp>).

An additional data set of 1,034,154 sequences, generated from *L. elliptica* mantle tissue using 454 technology, was provided by the British Antarctic Survey (BAS); for details see Clark et al 2010. After cleaning as described above, 808,460 sequences remained. The combined 1,298,384 reads of the first (ICMB) and second (BAS) dataset were mapped against the previous generated contigs using AMOScmp. The resulting contigs and singletons were then repeatedly assembled using TGICL (Cap3) software as described above. Finally all reads were mapped again against the assembled contigs using AMOScmp software. This resulted in the final contig dataset.

RNA sequence database analysis*RNA sequence database annotation and identification of candidate genes*

Putative gene names were assigned to the assembled contigs by BLASTx analysis against the UniprotKB Swissprot protein database (UniProt Knowledgebase, <http://www.expasy.org/sprot>), and tBLASTx against the NCBI non-redundant protein (nr) database (<http://www.ncbi.nlm.nih.gov>) with $e \leq 10^{-3}$ as cut off value. Conserved protein domains were identified using InterProScan (Hunter et al. 2009).

Annotation data were loaded together with contig sequence information into T-ACE, a software tool for processing of large RNA sequence data sets of non-model organisms

(Philipp et al. 2012a). Candidate genes for metabolic-, injury-, immune- and general stress response in *L. elliptica* were then identified by key word searches and sequence similarity searches in the annotated database. Candidate contigs were manually verified against the NCBI nr/nt and UniProtKB/Swissprot database using tBLASTx and Blastx, respectively. The protein domain structure of the different contigs was further investigated using the NCBI conserved domain architecture retrieval tool cDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) and compared to the conserved domain structure for the orthologous gene as defined by HomoloGene (<http://www.ncbi.nlm.nih.gov/homologene>).

Gene expression analysis

Expression changes of selected candidate genes were investigated in hemocytes and siphon tissue of starved and/or injured *L. elliptica* individuals by quantitative *real time* PCR (qRT-PCR) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) with SYBR Green (Power SYBR Green PCR Master Mix, Applied Biosystems, USA). Additionally, theromacin, a putative novel antimicrobial peptide in *L. elliptica*, was investigated using a tissue panel of large (mean and SD: 7.5 ± 0.5 cm), untreated *L. elliptica* individuals consisting of mantle, siphon, foot, gill, digestive gland and hemocytes (N=5-6). Tissues were ground in liquid nitrogen and total RNA extracted either via the standard TRI reagent protocol (Sigma-Aldrich, St. Louis, USA) in combination with Qiagen QiaShredder columns (Qiagen, Hilden, Germany), followed by clean-up using the Qiagen RNeasy columns (hemocyte cells, mantle, siphon and foot) or directly using an RNeasy kit in combination with QiaShredder columns (digestive gland and gills). All according to manufacturers' instructions and including DNase digestion steps. The quality and quantity of the extracted mRNA was checked as described above, and only samples showing 260/280 nm absorbance ratios of >2.1 were used for cDNA generation with the Advantage RT-for PCR Kit (Clontech, Heidelberg). Primers targeting candidate genes were designed using Primer3 (Rozen and Skaletsky 2000) and checked for hairpins, self-dimers and hetero-dimers with OligoAnalyzer 3.1 (Integrated DNA Technologies, USA) (Table 1). To ensure primer specificity and verify contig assembly, semi-quantitative PCR was performed with Advantage Taq2 polymerase (Clontech, Heidelberg) and the appropriate PCR conditions. The presence and size of PCR products was confirmed via agarose gel electrophoresis. The cDNA was isolated from the gel with the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and Sanger sequenced using Big Dye Terminator chemistry (Applied Biosystems, USA). The resulting Sanger sequences were compared with

the respective contig from the RNA sequence database using Sequencher version 4.5 (GeneCodes, Ann Arbor, MI, USA). qRT-PCR analysis for each sample was performed in triplicate. The conditions were as follows: 50°C (2 min), 95°C (10 min), 45 cycles of 95°C (15s) and 60°C (1min). To check for artifacts, a melting curve was generated for each primer pair. The comparative CT method (delta Ct) for the relative quantification of gene expression was used (Livak and Schmittgen 2001) and data were normalized using 18s ribosomal RNA (18s rRNA) as the reference gene, which itself was not found to be affected by treatment or tissue.

Tab. 1: Primer data for quantitative *real time* PCR analysis of selected candidate genes involved in the injury, immune system and general stress responses. The contig ID of the *Laternula elliptica* RNA sequence database, reverse (R) and forward (F) primer sequences and amplicon length are given for each candidate gene.

Gene name	Primer name	Accession number	Amplicon length [bp]	Primer sequence
18S rRNA	<i>Le</i> -18S rRNA_F	HE804712	352	CACCACCAACCACCGAAT
	<i>Le</i> -18S rRNA_R			AAGACGAACGACAGCGAAAG
Actin	<i>Le</i> -Actin_F	HE804713	375	GCCAAATCCAGACGAAGG
	<i>Le</i> -Actin_R			TGAAGCCCAGAGTAAGAGAGG
Catalase	<i>Le</i> -Catalase_F	HE804714	378	CATTCTCTCCCGCACACAT
	<i>Le</i> -Catalase_R			GCCTTTCACGCTCCTCTG
Chitinase	<i>Le</i> -Chitinase_F	HE804715	223	TGCCAAAAGAAAATGCTGATT
	<i>Le</i> -Chitinase_R			GCCGTTGTTGAGTGTGCTAA
HSP70	<i>Le</i> -HSP70_F	HE804716	394	TCTCTCACCTCCAACCTTCTT
	<i>Le</i> -HSP70_R			TCCATCAAACACAATCTTCG
SOD [CuZn]	<i>Le</i> -SOD[CuZn]_F	HE804717	305	ACACCCAACGACTACCATCC
	<i>Le</i> -SOD[CuZn]_R			CGGCTACCACATCCACAAG
Theromacin	<i>Le</i> -Theromacin_F	HE804718	337	GCAGCCCACAGAGCAGTAAC
	<i>Le</i> -Theromacin_R			TAAAAGCGGACACCACCAT
TIMP	<i>Le</i> -TIMP_F	HE804719	322	CTGGGATTGCTTGCTGTGTA
	<i>Le</i> -TIMP_R			TCTGGGTCTGGATGAAAACA

Statistical analysis

Gene expression data sets and data of the hepatosomatic index were tested for Gaussian distribution using the Kolmogorov-Smirnov-test. Homogeneity of variances was assessed by the Brown-Forsythe-Test for equal variances. When necessary, data were transformed (square-root, log, LN or $-1 * \log Y$) to achieve normal distribution and homogeneity of variances.

In case of normally distributed data, statistical analysis was performed using the software package STATISTICA for Windows version 7.1 (StatSoft Inc., USA). Effects of time and treatment were evaluated by two-way-ANOVA and the Bonferroni posthoc test.

In case of non-normally distributed data these were analyzed using the non-parametric “adonis” PERMANOVA (Permutational Multivariate Analysis of Variance using distance matrices) (Anderson 2001) of the R software package “vegan” (Dixon 2003; Oksanen et al. 2011), R statistic software version 2.13.1.

Data sets which did not show equal variances were analyzed performing t-tests corrected for non-equal variances (Welch correction) offered by the Software GraphPad Prism version 5.01 for Windows (Graphpad Software, USA).

Tissue-specific differential expression of theromacin was determined by one-way-ANOVA after assessing the homogeneity of variances using Bartlett’s test.

Results

RNA sequence database overview

RNA sequence data were generated from pooled cDNA samples of hemocytes, digestive gland, gill and siphon tissue of stress treated *L. elliptica* individuals using high-throughput sequencing (GS FLX, Roche-454 Life Sciences). 489,924 sequences generated at the ICMB, together with 808,460 sequences provided by BAS, were assembled into 42,525 contigs (average size: 749bp, N50: 877) and 114,322 singletons. The GC-content of the contigs was 38.94% (AT/GC-ratio: 1.56). After BLAST annotation 20,409 contigs were found to show high sequence similarity to previously published sequences from other organisms. 618 of the contigs were annotated by already published sequences from *L. elliptica*. 12,171 contigs contained conserved protein domains. An overview of the RNA sequence database annotation is given in Table 2.

Tab. 2: Summary of *Laternula elliptica* contig annotation.

	All sequences (≥ 100 bp)	100-500bp	>500bp
Total number of contigs	41964	15741	26223
with BLAST matches	20409	5628	14781
with assigned GO terms	8934	1885	7049
with InterProScan matches	12171	2560	9611
without matches	20985	9932	11053
% annotated	49.99	36.90	57.85

Using key word searches, a high number of contigs, with sequence similarity to known genes potentially involved in the *Laternula elliptica* stress defense and immune reactions were identified (Table 3, supplementary Table S7). These included matches with high sequence similarity to functional components of the innate immune system such as the pro-inflammatory cytokine interleukin 17 (IL17), members of the NADPH oxidase (NOX)/ Dual oxidase (DUOX) family, cellular recognition receptors (toll like receptors) or antimicrobial peptides (theromacin, mytilin, mytimycin). Further contigs with high sequence similarity to genes known to be involved in oxidative stress defense, general stress responses and oxygen sensing (heat-shock proteins, hypoxia inducible factor, prolyl 4-hydroxylase), detoxification (cytochrome p450) and autophagic processes (autophagy related proteins) were identified. All putative proteins encoded by these contigs are potentially involved in the maintenance of cellular homeostasis, and contigs with putative functions for oxidative stress defense (copper-zinc superoxide dismutase, Cu/Zn SOD; catalase, Cat), general stress response (heat-shock protein 70, HSP70), tissue regeneration and development (chitinase, actin), antimicrobial defense (theromacin) and response to injury and bacterial infection (tissue inhibitors of metalloproteinases, TIMP) were selected for detailed investigation into gene expression changes related to starvation and injury treatments.

The RNA sequence database project was deposited as sequence read archive (SRA) study ERP001323 and project number PRJEB33 at the European Nucleotide Archive (ENA). Assembled RNA sequences were deposited in the ENA as Transcriptome Shotgun Assembly (TSA) and accession numbers are given throughout the text or tables.

Tab. 3: Selected contigs putatively involved in the *L. elliptica* stress response and immune reaction. The information regarding the best BLAST match within the UniprotKB/Swissprot and NCBI/nt-blast databases is given for each *L. elliptica* contig. Further contig details including sequence length and domain structure are listed in supplementary table 8. Genes/contigs marked with * were investigated for differential expression in stress treated *L. elliptica* individuals by qRT-PCR.

Category/ Gene name	<i>L. elliptica</i> contig Accession	Best blast hit UniprotKB/Swissprot				Best blast hit NCBI, nt			
		Accession	Description	Organism	E-value	Accession	Description	Organism	E-value
<i>Oxidative stress defense</i>									
Alternative oxidase	HE804720	Q9P959	Alternative oxidase, mitochondrial	<i>Emericella nidulans</i>	8E-67	FJ177509.1	Alternative oxidase	<i>Crassostrea gigas</i>	3E-142
Catalase	HE804714*	P00432	Catalase	<i>Bos taurus</i>	0	HM147935.1	Catalase (Cat-2)	<i>Crassostrea hongkongensis</i>	0
Glutathione peroxidase	HE804721	P37141	Glutathione peroxidase 3	<i>Bos taurus</i>	4E-37	DQ830766.1	Selenium-dependent glutathione peroxidase	<i>Unio tumidus</i>	2E-54
Peroxiredoxin 5	HE804722	Q9BG11	Peroxiredoxin-5, mitochondrial	<i>Bos taurus</i>	2E-37	EU734750.1	Peroxiredoxin V	<i>Laternula elliptica</i>	0
Peroxiredoxin 6	HE804723	Q5ZJF4	Peroxiredoxin-6	<i>Gallus gallus</i>	3E-76	EU734751.1	Peroxiredoxin VI	<i>Laternula elliptica</i>	0
Superoxide dismutase (Cu/Zn)	HE804717*	P10792	Superoxide dismutase (Cu-Zn), chloroplastic	<i>Petunia hybrida</i>	1E-07	XM_002734238.1	Predicted: superoxide dismutase-like protein	<i>Saccoglossus kowalevskii</i>	2E-118
Superoxide dismutase (Mn)	HE804724	P07895	Superoxide dismutase (Mn), mitochondrial	<i>Rattus norvegicus</i>	2E-78	GQ202272.1	Manganese superoxide dismutase	<i>Laternula elliptica</i>	0
<i>Immune response</i>									
Allograft inflammatory factor	HE804725	P55008	Allograft inflammatory factor 1	<i>Homo sapiens</i>	3E-36	GQ384410.1	Allograft inflammatory factor	<i>Venerupis philippinarum</i>	2E-50
Beta-glucan recognition protein	HE804726	Q8N0N3	Beta-1,3-glucan-binding protein	<i>Penaeus monodon</i>	1E-101	JF309105.1	Beta-glucan recognition protein	<i>Tapes literata</i>	0
Chitinase	HE804715*	Q91XA9	Acidic mammalian chitinase	<i>Mus musculus</i>	1E-111	AJ971239.1	Chit3 protein	<i>Crassostrea gigas</i>	1E-151
Defensin	HE804727	P80571	Defensin MGD-1	<i>Mytilus galloprovincialis</i>	2E-06	DQ864986.1	Amercin	<i>Amblyomma americanum</i>	4E-08
Dual oxidase	HE804728	Q9NRD8	Dual oxidase 2	<i>Homo sapiens</i>	6E-26	XM_001111340.2	Predicted: dual oxidase 1	<i>Macaca mulatta</i>	2E-27
	HE804729	Q8HZK2	Dual oxidase 2	<i>Sus scrofa</i>	0	NM_001204642	thyroid peroxidase-like protein (LOC100533347)	<i>Aplysia californica</i>	1E-28
Interleukin 17	HE804730	A9XE49	Interleukin 17-like protein	<i>Crassostrea gigas</i>	4E-07	XM_002932858.1	Predicted: interleukin-17D-like	<i>Xenopus (Silurana) tropicalis</i>	6E-06

Macrophage migration inhibitory factor	HE804731	A9JSE7	Macrophage migration inhibitory factor	<i>Xenopus tropicalis</i>	1E-20	JN564748.1	Macrophage migration inhibitory factor	<i>Mytilus galloprovincialis</i>	3E-38
Mytilin	HE804732	P81613	Mytilin-B	<i>Mytilus edulis</i>	4E-16	AF162336	Mytilin B antimicrobial peptide precursor	<i>Mytilus galloprovincialis</i>	1E-91
	HE804733	P81613	Mytilin-B	<i>Mytilus edulis</i>	8E-10	AY730626.1	Mytilin C precursor	<i>Mytilus trossulus</i>	1E-68
Mytimycin	HE804734	P81614	Mytimycin	<i>Mytilus edulis</i>	4E-07	FJ804478.1	Mytimycin precursor	<i>Mytilus galloprovincialis</i>	8E-52
NADPH oxidase	HE804735	Q96PH1	NADPH oxidase 5	<i>Homo sapiens</i>	1E-124	XM_002731038.1	Predicted: NADPH oxidase, EF-hand calcium binding domain 5-like	<i>Saccoglossus kowalevskii</i>	3E-172
Nitric oxide synthase	HE804736	Q9Z0J4	Nitric oxide synthase, brain	<i>Mus musculus</i>	1E-80	AB333805.1	Nitric oxide synthase	<i>Lehmannia valentiana</i>	4E-101
Nuclear factor NF-kappa-B	HE804737	O73630	Nuclear factor NF-kappa-B p100 subunit	<i>Xenopus laevis</i>	1E-44	NM_001165001.1	Nuclear factor kappa-B	<i>Saccoglossus kowalevskii</i>	1E-49
	HE804738	P25799	Nuclear factor NF-kappa-B p105 subunit	<i>Mus musculus</i>	4E-61	NM_214654.1	NFκB protein	<i>Strongylocentrotus purpuratus</i>	8E-96
Peptidoglycan-recognition protein	HE804739	Q70PU1	Peptidoglycan-recognition protein SC2	<i>Drosophila simulans</i>	7E-40	AB425335.1	Peptidoglycan recognition protein S1S	<i>Crassostrea gigas</i>	1E-89
Tissue inhibitor of metalloproteinase	HE804719*	Q9JHB3	Metalloproteinase inhibitor 4	<i>Mus musculus</i>	1E-18	AK079347.1	Tissue inhibitor of metalloproteinase 4	<i>Mus musculus</i>	1E-11
TNF receptor associated factor	HE804740	P39429	TNF receptor-associated factor 2	<i>Mus musculus</i>	1E-107	XM_003423039.1	Predicted: TNF receptor-associated factor 2	<i>Loxodonta africana</i>	8E-103
Toll like receptor	HE804741	Q9GL65	Toll-like receptor 4	<i>Bos taurus</i>	1E-35	XM_003217353.1	Predicted: toll-like receptor 13-like	<i>Anolis carolinensis</i>	9E-27
	HE804742	Q6R5N8	Toll-like receptor 13	<i>Mus musculus</i>	1E-19	XM_003217353.1	Predicted: toll-like receptor 13-like	<i>Anolis carolinensis</i>	1E-22
	HE804743	Q9EPW9	Toll-like receptor 6	<i>Mus musculus</i>	3E-16	HM215599.1	Toll-like receptor 2	<i>Arvicola terrestris</i>	5E-12
Hydramacin, Theromacin	HE804718*	B3RFR8	Hydramacin-1	<i>Hydra vulgaris</i>	4E-14	HM598084.1	Theromacin	<i>Hyriopsis cumingii</i>	9E-29
Stress response									
Chaperonin	HE804744	Q64433	10 kDa heat shock protein, mitochondrial	<i>Mus musculus</i>	5E-35	XM_003434292.1	Predicted: 10 kDa heat shock protein, mitochondrial-like	<i>Canis lupus</i>	4E-41
DnaJ	HE804745	Q8WW22	Isoform 2 of DnaJ homolog subfamily A member	<i>Homo sapiens</i>	1E-137	NM_001079380.1	DnaJ (Hsp40) homolog	<i>Xenopus (Silurana) tropicalis</i>	4E-139
HSP, low molecular weight	HE804746	P02518	Heat shock protein 27	<i>Drosophila melanogaster</i>	2E-13	GQ384407.1	Heat shock protein 22 isoform 1	<i>Venerupis philippinarum</i>	8E-96
HSP60	HE804747	P18687	60 kDa heat shock protein, mitochondrial	<i>Cricetulus griseus</i>	0	FJ480412.1	Heat shock protein 60	<i>Biomphalaria glabrata</i>	0
HSP70	HE804716*	Q9U639	Heat shock 70 kDa protein cognate 4	<i>Manduca sexta</i>	0	EF198332.1	Heat shock protein 70	<i>Laternula elliptica</i>	0
HSP90	HE804748	O02705	Heat shock protein HSP 90-alpha	<i>Sus scrofa</i>	0	EU831278.1	Heat shock protein 90	<i>Laternula elliptica</i>	0

Hypoxia inducible factor	HE804749	Q9Y1B9	Hypoxia-inducible factor 1 alpha	<i>Gallus gallus</i>	2E-93	AB289857.1	Hypoxia-inducible factor 1 alpha	<i>Crassostrea gigas</i>	3E-111
	HE804750	Q98SW2	Hypoxia-inducible factor 1 alpha	<i>Oncorhynchus mykiss</i>	8E-04	AB289857.1	Hypoxia-inducible factor 1 alpha	<i>Crassostrea gigas</i>	5E-44
Prolyl 4-hydroxylase	HE804751	P13674	Prolyl 4-hydroxylase subunit alpha-1	<i>Homo sapiens</i>	7E-89	XM_002429598.1	Prolyl 4-hydroxylase alpha-1 subunit precursor	<i>Pediculus humanus</i>	6E-108
Thioredoxin	HE804752	O96952	Thioredoxin	<i>Geodia cydonium</i>	1E-20	AY652616.1	Thioredoxin	<i>Chlamys farreri</i>	6E-27
Cellular homeostasis, detoxification, metabolism									
Autophagy related protein 2	HE804753	Q96BY7	Autophagy-related protein 2 homolog B	<i>Homo sapiens</i>	2E-52	XM_001489195.3	Predicted: ATG2 autophagy related 2 homolog B	<i>Equus caballus</i>	2E-58
Autophagy related protein 3	HE804754	Q9CPX6	Ubiquitin-like-conjugating enzyme ATG3	<i>Mus musculus</i>	5E-51	NM_001142489.1	Autophagy related protein Atg3-like protein	<i>Bombyx mori</i>	5E-59
	HE804755	Q6PFS7	Ubiquitin-like-conjugating enzyme ATG3	<i>Danio rerio</i>	3E-61	XM_001601049.2	Predicted: autophagy-related 3-like	<i>Nasonia vitripennis</i>	2E-69
Autophagy related protein 4	HE804756	Q6PZ02	Cysteine protease ATG4B	<i>Gallus gallus</i>	1E-110	AB513350.1	ATG4 mRNA for autophagy-related 4	<i>Haemaphysalis longicornis</i>	1E-121
	HE804757	Q68FJ9	Cysteine protease ATG4D	<i>Xenopus laevis</i>	4E-31	XM_001181425.1	Predicted: similar to autophagy-related 4D	<i>Strongylocentrotus purpuratus</i>	7E-40
Autophagy related protein12	HE804758	O94817	Ubiquitin-like protein ATG12	<i>Homo sapiens</i>	2E-31	XM_002939961.1	Predicted: autophagy-related protein 12-like (LOC100493403)	<i>Xenopus (Silurana) tropicalis</i>	7E-35
Cytochrome p450	HE804759	Q9HCS2	Cytochrome P450 4F12	<i>Homo sapiens</i>	3E-85	HM126463.1	Cytochrome P450 family 4 protein	<i>Perinereis aibuhitensis</i>	5E-109
	HE804760	Q92113	Cytochrome p450 family: steroid 17-alpha-hydroxylase/17,20 lyase	<i>Squalus acanthias</i>	8E-26	EF451959.1	cytochrome P450-related protein	<i>Crassostrea gigas</i>	2E-38
Ferritin	HE804761	P42577	Soma ferritin	<i>Lymnaea stagnalis</i>	3E-70	GQ139542.1	Ferritin-like protein	<i>Pinctada maxima</i>	7E-87
Glutathione S-transferase	HE804762	P19157	Glutathione S-transferase P 1	<i>Mus musculus</i>	1E-48	EU131183.1	Pi class glutathione S-transferase	<i>Laternula elliptica</i>	0
Metallothionein	HE804763	P80247	Metallothionein 10-II	<i>Mytilus edulis</i>	7E-27	DQ832722.1	Metallothionein 10a	<i>Laternula elliptica</i>	7E-42
Housekeeping genes									
Actin	HE804713*	P12716	Actin, cytoplasmic	<i>Pisaster ochraceus</i>	0	JN084197.1	Beta-actin	<i>Meretrix meretrix</i>	0
18s rRNA	HE804712*	Q9Y4H2	Insulin receptor substrate 2	<i>Homo sapiens</i>	1E-02	AY192687.1	18S ribosomal RNA	<i>Laternula elliptica</i>	0

Tissue expression profiling of Laternula elliptica theromacin mRNA

Theromacin has been putatively designated as a novel antimicrobial peptide. As a first step towards further characterizing this transcript and defining functionality, tissue-specific expression was performed in *Laternula elliptica*. The relative mRNA expression of *Le-theromacin* was determined in hemocytes, digestive gland, gill, mantle, siphon and foot of untreated, large individuals. Gene expression was detected in all tissues (Figure 1). The highest expression was found in mantle, siphon and foot tissue, lower values in gill and digestive gland tissue and lowest expression in hemocytes, which had 193-fold lower values compared to the highest expression levels in the mantle tissue.

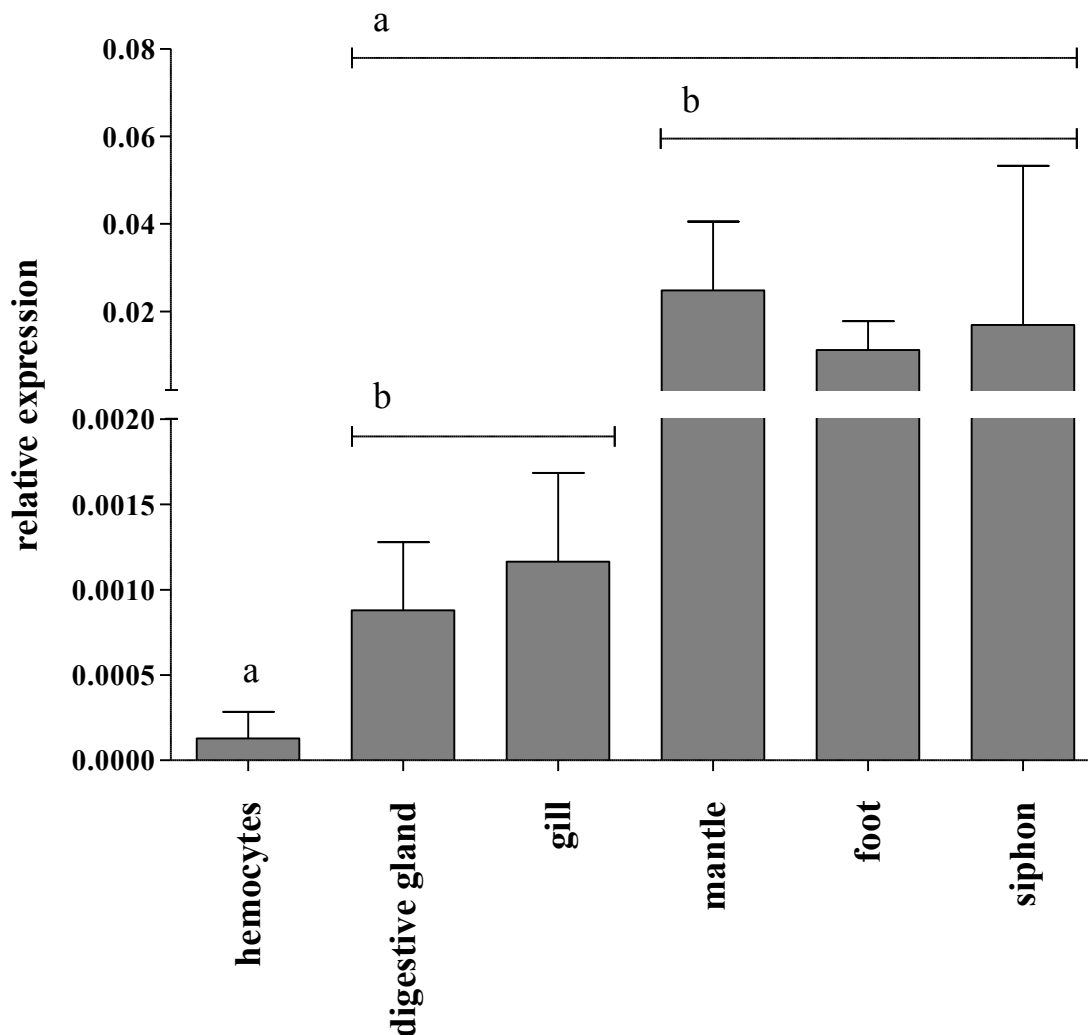


Fig. 1: *L. elliptica* mRNA expression levels of *Le-theromacin* in different tissues and hemocytes analysed by qRT-PCR. Values were normalized using 18s ribosomal RNA as the reference gene. Data are presented as median and interquartile ranges of relative expression values, N=5 per group (hemocytes: N=6). Columns, or groups of columns, with similar letters are significantly different from each other.

Effect of injury and starvation on hemocyte and siphon gene expression

Expression levels of candidate genes putatively involved in the immune and general stress responses of *L. elliptica* were investigated under injury and starvation in a size and time dependent manner. Two tissues were analysed, hemocytes because they are major components of the immune system and siphon as it was directly inflicted with injury in the experiment.

The most prominent effect was observed in the hemocytes of the small individuals (Figure 2, supplementary Table S1 and S3). After acute injury (2 days), expression levels of *Le*-TIMP, *Le*-SOD, *Le*-HSP70, *Le*-catalase and *Le*-chitinase increased significantly (>10fold) compared to non-injured individuals in the fed group. In contrast, small animals which were starved for 21 days prior the wound infliction did not respond with increased gene expression levels. A similar pattern of enhanced gene expression was observed in large injured individuals, but the response was less pronounced and induced levels did not reach significance. After three weeks of recovery and repair from the injury impact, gene expression levels were back to control levels in both size groups (Figure 2).

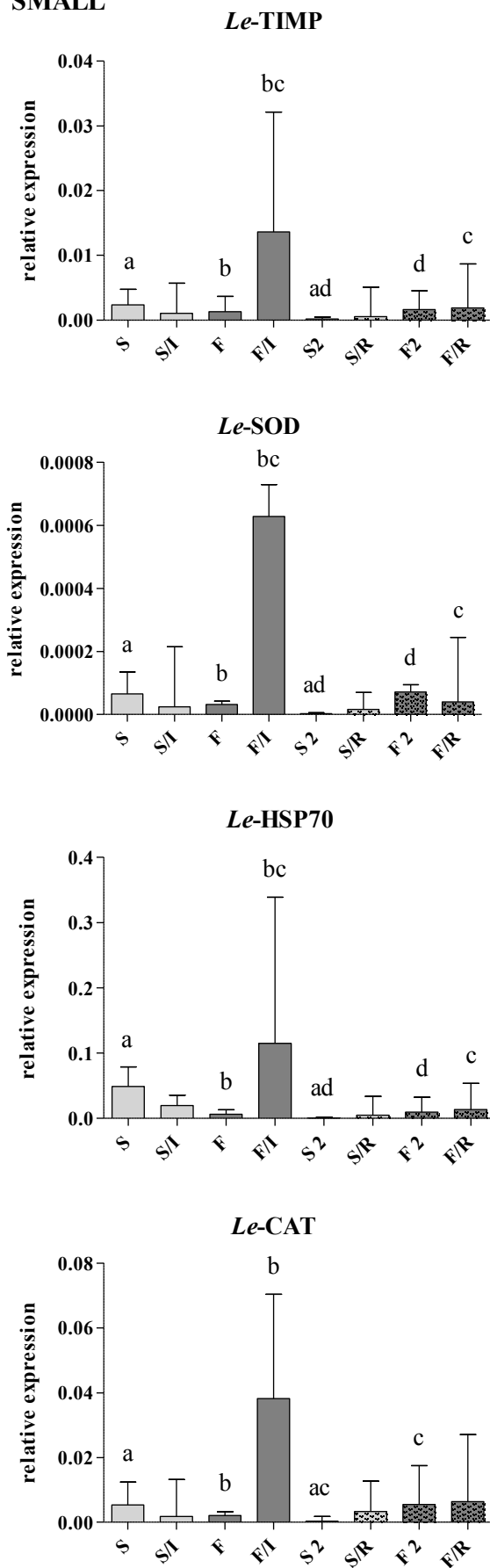
Overall, feeding conditions had no significant effect on hemocyte gene expression in non-injured individuals. The response of different genes was, however, not uniform and minor trends were detectable. After the first three weeks of treatment, the majority of the genes under investigation appeared to be less highly expressed in fed compared with starved animals. This was especially the case for *Le*-TIMP, *Le*-SOD, *Le*-HSP70 and *Le*-catalase. After three additional weeks of food administration and starvation treatments, the reverse pattern was observed, with higher expression levels in fed compared with starved individuals (Figure 2). This was mainly due to a decrease in expression levels under the long-term starvation treatment, whereas gene expression levels in the food treatment remained constant throughout the experiment. Again, responses were more pronounced in small compared with larger individuals.

In the siphon tissue, gene expression differed only slightly between the food treatment and injury groups (Figure 3, supplementary Table S2 and S4). For *Le*-TIMP, *Le*-SOD, *Le*-HSP70 and *Le*-actin a slight increase in expression levels after acute injury was observed in both size groups of starved and fed animals. In most cases these changes failed to reach significance. In contrast to the results in hemocytes, “time” had a strong effect in siphon gene expression. In both size and food/starvation groups, expression levels increased between the first and second sampling date in *Le*-TIMP, *Le*-SOD, *Le*-catalase, *Le*-chitinase, *Le*-HSP70 and *Le*-actin.

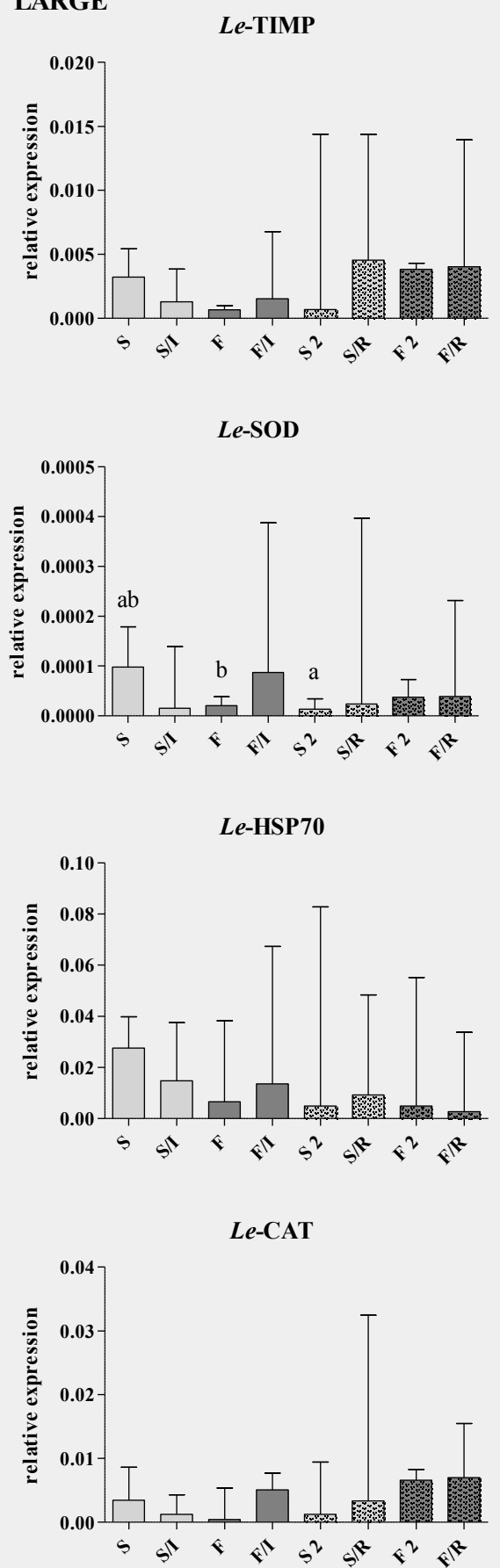
Effect of injury and starvation on the hepatosomatic index (HSI)

Feeding conditions as well as injury infliction had no significant effect on the HSI in small individuals (supplementary Table S5 and S6). The HSI of large individuals was significantly affected by the feeding conditions. Non-injured animals had a lower HSI after three weeks of starvation conditions (median: 6.56), compared to non-injured animals after 3 weeks of food supply (median: 7.82, significantly different after Bonferroni post hoc test). The HSI in injured animals was not affected after either three or six weeks of starvation treatment (supplementary Table S5 and S6).

SMALL



LARGE



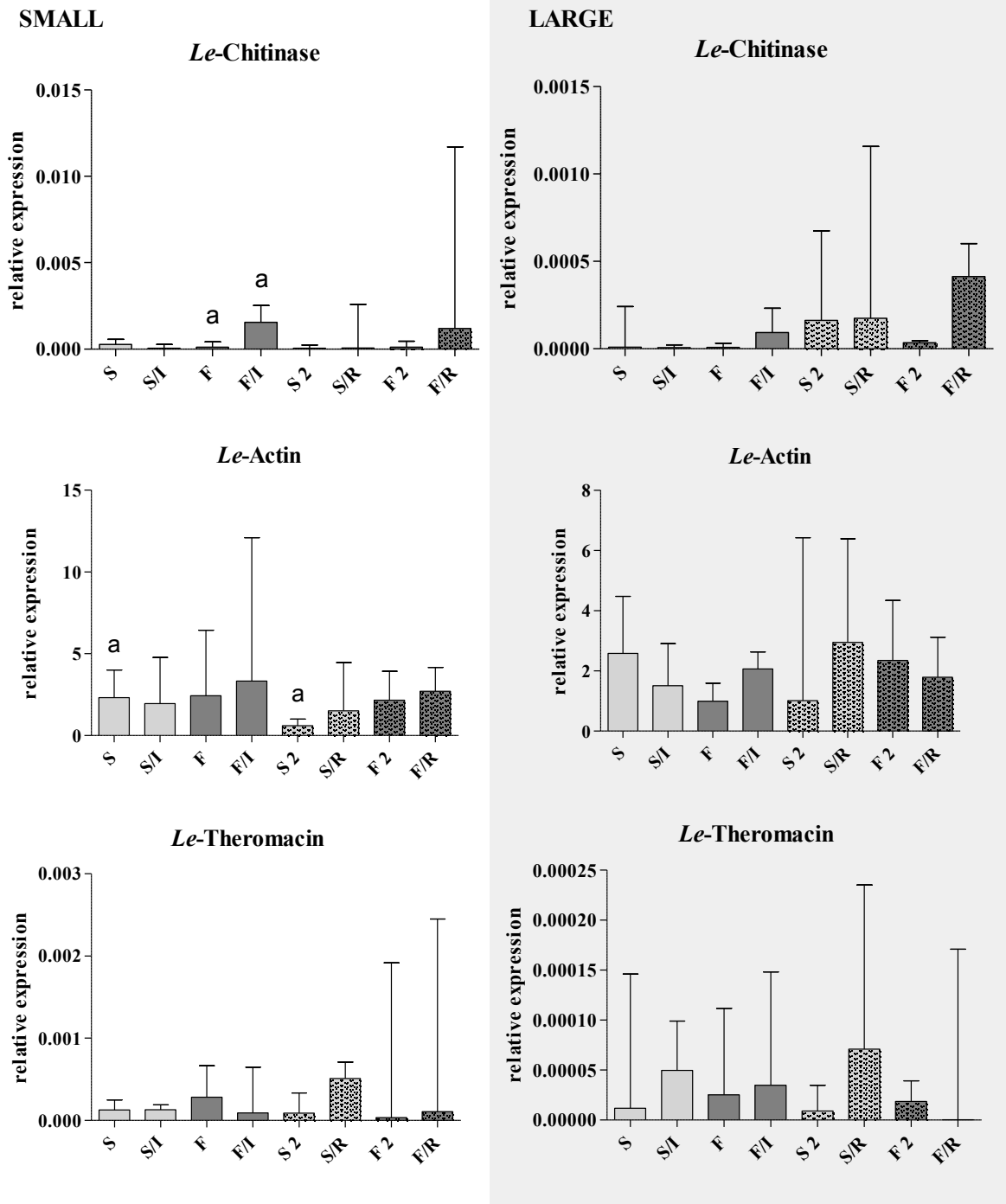
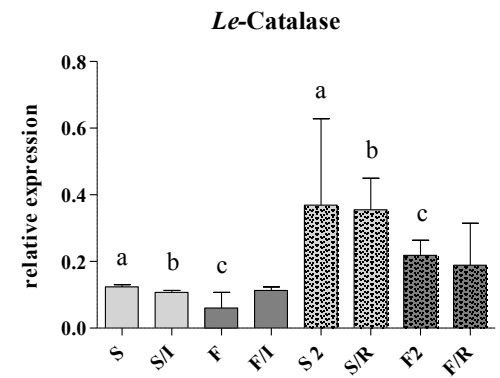
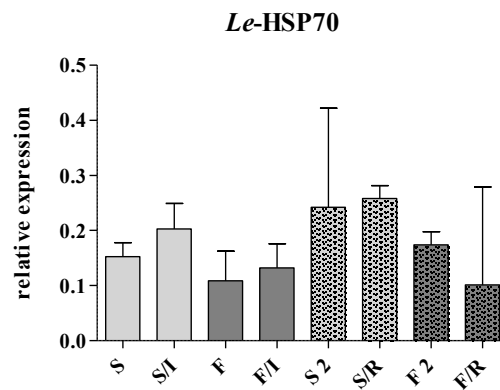
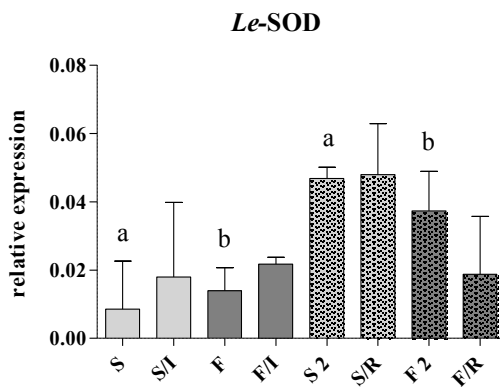
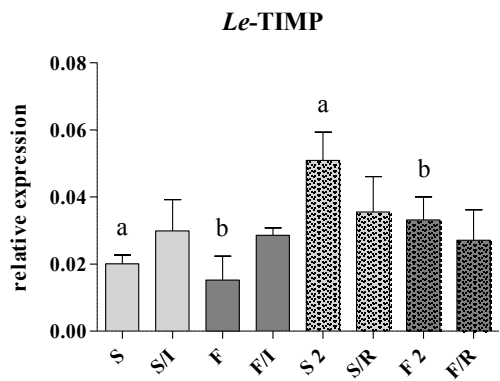
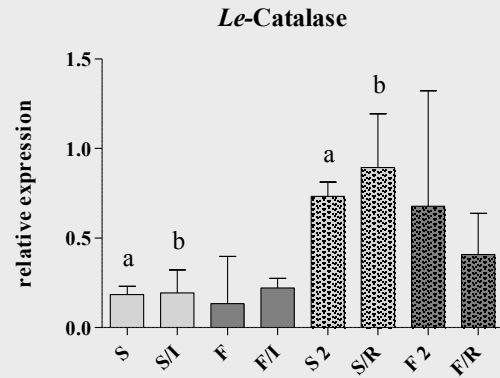
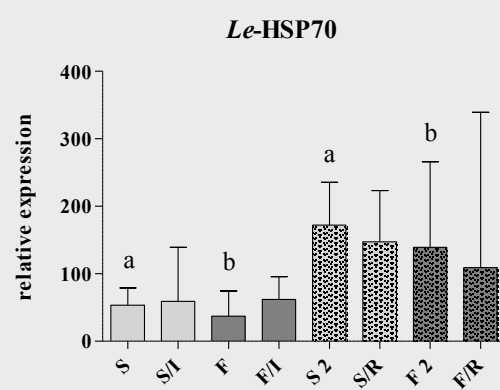
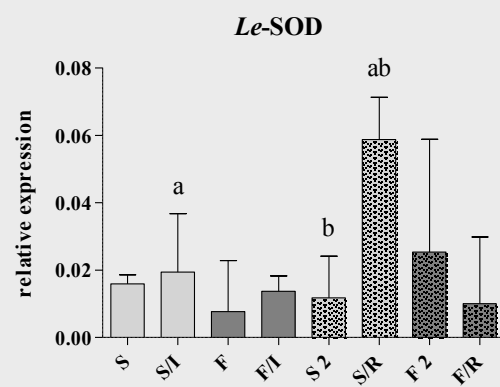
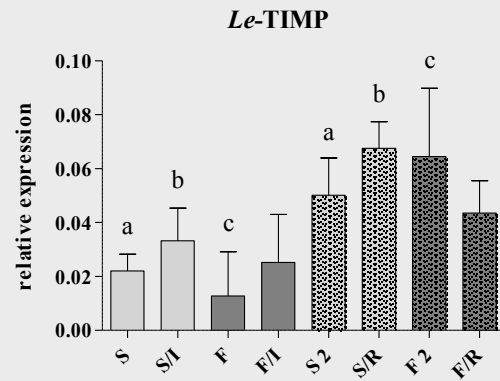


Fig. 2: *L. elliptica* hemocytes: Expression levels of selected contigs putatively involved in the stress and immune responses measured by qRT-PCR in different *Laternula elliptica* size/age groups individuals (small, large) sampled after 23 days of “food” (F) and “starvation” (S) treatment and experiencing acute (2 days after infliction) injury (S/I and F/I). Additional samples were taken after 44 days of “food” (F 2) and “starvation” (S 2) and recovering for 21 days from the previous inflicted injury (S/R and F/R). For experimental details see materials and methods section. Expression levels were normalized using the 18s ribosomal RNA as the reference gene. Data are presented as the median and interquartile range. N=3-10 individuals per group. Groups with similar letters are significantly different from each other with $p < 0.05$ (two-way ANOVA with Bonferroni post hoc test). Significant differences of *Le*-SOD expression levels between the groups of small individuals were identified using an unpaired t-test with Welch correction for unequal variances or the non-parametric Mann-Whitney test.

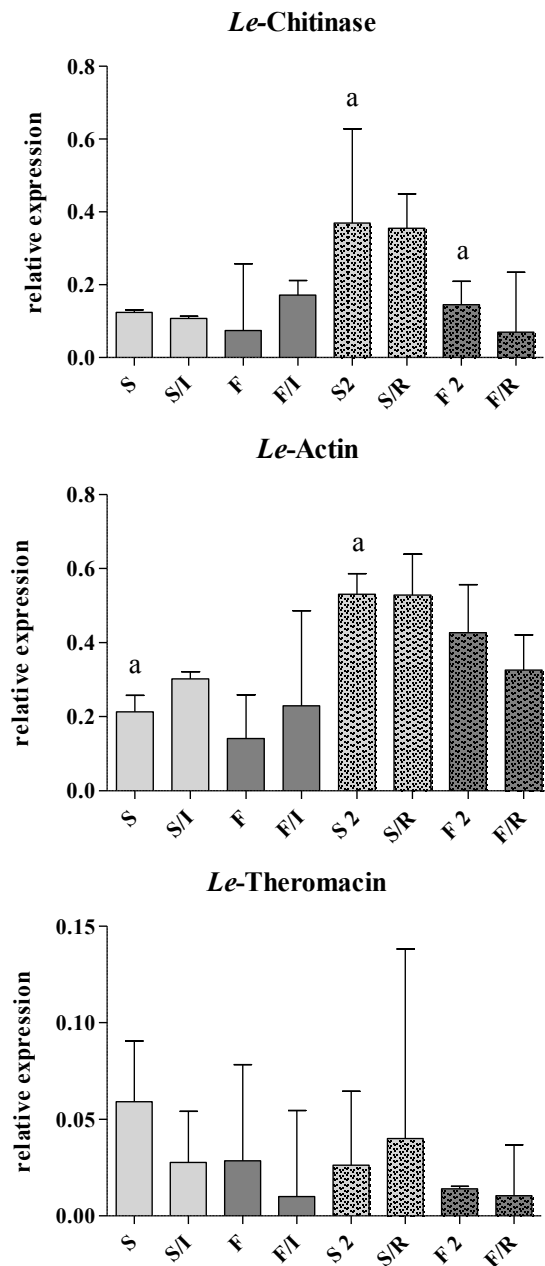
SMALL



LARGE



SMALL



LARGE

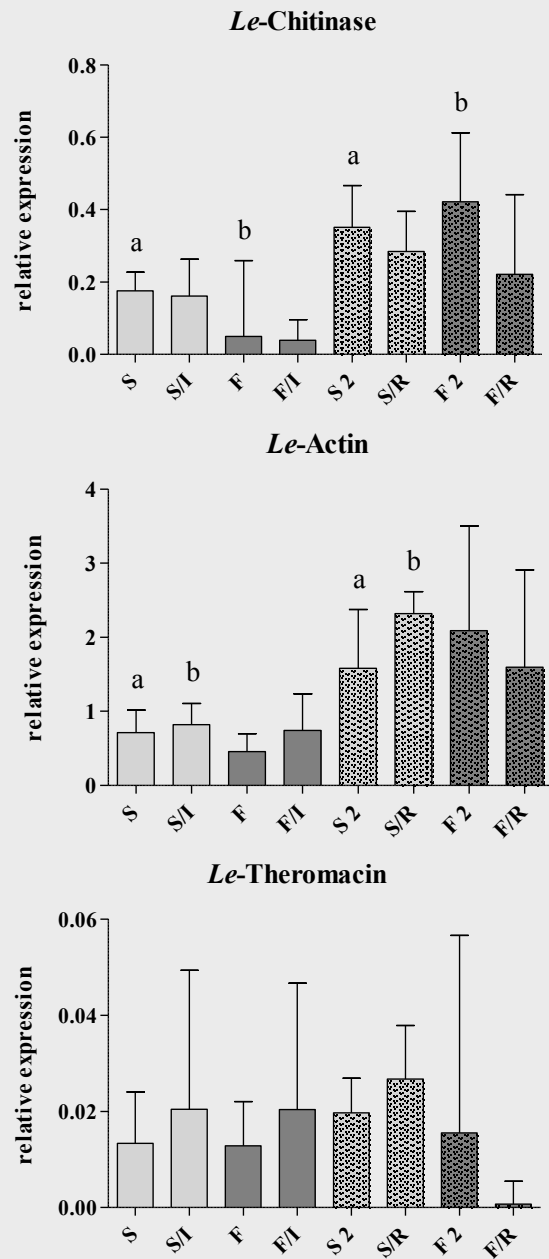


Fig. 3: Siphon gene-expression levels measured by qRT-PCR in different *L. elliptica* size/age groups (small, large) sampled after 23 days of “food” (F) and “starvation” (S) treatment and experiencing acute (2 days since infliction) injury (S/I and F/I). Additional samples were taken after 44 days of “food” (F 2) and “starvation” (S 2) and recovering (S/R and F/R) from the previous inflicted injury (for details see material and method section). Expression levels were normalized using the 18s ribosomal RNA as the reference gene. Data are presented as the median and interquartile ranges. N=5-9 individuals per group. Groups with similar letters are significantly different from each other with $p < 0.05$ (two-way ANOVA with Bonferroni post hoc test).

Discussion

Identification of genes involved in immunity

To enhance the amount of sequence information of genes involved in the stress response and immunity in bivalves, an RNA sequence database was generated from hemocyte and tissue samples of immune-stimulated *L. elliptica*. Half of the assembled contigs of *L. elliptica* could not be annotated using sequence similarity searching to known genes from other organisms or structurally characterized by InterProScan. This highlights the sparsity of sequence and functional information existing for marine bivalves and, in particular, *L. elliptica*. Nevertheless, the resulting contigs provide an excellent resource for the discovery of putative genes involved in immunity and/or tissue repair in *L. elliptica* and will further help to identify such genes in other bivalves.

Components of the innate immune system identified in the *L. elliptica* database included cellular receptors, signal transduction molecules, effector proteins such as antimicrobial peptides as well as components of detoxification, oxidative stress regulation, protein stabilization (HSPs) or cytoskeleton regeneration. These genes are important in the maintenance of homeostasis under stressful conditions and enable the animal to support an effective cellular response to environmental disturbance. Seven genes were selected from the database, which have previously been reported to play a role in the stress response, especially under starvation and injury, in other organisms. These were used to conduct a functional analysis in the Antarctic bivalve *L. elliptica*.

Cross effects of starvation and injury on gene expression in hemocytes and siphon tissue

The expression of the selected antimicrobial and oxidative stress genes after injury in large individuals was not significantly influenced by the feeding conditions. The lack of change in gene expression under “starvation”, especially in large individuals, may be due to treatment time. In fact, *L. elliptica* seemed not to be critically starved, although they received no food for 3 weeks. This was substantiated by results in the young individuals. These animals have a higher metabolic activity and although there was an overall reduction in stress gene expression during the experimental period, this was not significant. The hepatosomatic index was not significantly affected in any treatment group of the small animals throughout the 3 week starvation experiment, whereas in large animals the HSI was significantly reduced by three weeks of starvation (Bonferroni post hoc test). There were, however, no significant effects of starvation in all other treatments. In particular, HSI was also not reduced in injured

animals after three weeks of starvation. Therefore, the reduced values in non-injured animals after three weeks of starvation may have been due to other, as yet unknown, factors and not solely due to starvation.

This lack of a response to 3 weeks starvation is almost certainly due to the adaptation of marine species to the cold Antarctic environment. Antarctic invertebrates such as the brachiopod *Liothyrella uva* were shown to have typically prolonged post-prandial elevation of metabolic rates (Peck 1998, 2002; Peck and Veal 2001) which points to a long duration of food utilization and may delay the onset of severe starvation in Antarctic invertebrates. In addition, Antarctic invertebrates typically have low metabolic rates as an adaptation to the extremely cold climate and intense seasonality with short summers and long periods of potential food shortage during winter (Clarke et al. 2008; Peck and Conway 2000; Schloss et al. 2012). In addition, the induction of stress response genes in Antarctic invertebrates is potentially attenuated in Antarctic species compared with comparable temperate invertebrates (see the tiered response of heat shock genes to tidal emersion in Antarctic and Patagonian limpets in Pöhlmann et al. (2011)).

In small animals, however, the gene transcription levels were generally higher in the hemocytes of fed compared with starved specimens following injury. Cell counts conducted in the same small animals showed that the number of hemocytes per ml of hemolymph only increased in small injured animals under starvation (Husmann et al. 2011). Thus, whereas starved animals enhanced the number of hemocytes to mitigate the effects of injury, fed specimens may enhance the immunocompetence of the individual cells by inducing the expression of anti-stress and repair genes. This is in keeping with the idea that maintenance of immunocompetence is energetically costly as it involves gene transcription and protein synthesis (Lochmiller and Deerenberg 2000). The magnitude of the inducible response hence depends on the energetic reserves and feeding state of each individual. The origin of the additional free floating hemocytes in starved and injured small bivalves is unclear.

Time dependent patterns of gene expression in control animals

All selected candidate genes showed basal expression levels in the hemocytes and siphon tissue of non-injured animals. The down-regulation of immune-related genes after 4 weeks starvation in non-injured *L. elliptica* may represent an adaptation to the decline of external stimuli in filtered seawater, as hemocytes respond directly to external stimuli such as bacteria. Increasing levels of most candidate genes in the siphon tissue of fed and starved individuals with time during the experiment possibly reflected a general adaptation to stress during

prolonged maintenance in the holding system when they were not in their natural environment of being buried in sediment.

Changes of gene expression in the hemocytes of injured animals

As a first step towards investigating the molecular defense mechanisms in *L. elliptica* which might be influenced by environmental stress in the Western Antarctic Peninsula, putative candidate genes that are potentially regulated by food-deprivation and injury were identified. The observation that injury had clearer effects on the up-regulation of candidate genes compared with food-deprivation treatments in hemocytes highlighted the involvement of these genes in the immune defense of *L. elliptica*. In contrast, food-deprivation had only minor effects on gene expression at least within the 4 weeks time frame, which may be too short to initiate starvation in Antarctic bivalves due to their low metabolic rates and their capacity to survive the intense seasonality of the Antarctic environment, as shown for other Antarctic marine invertebrates (Peck and Conway 2000; Peck 1998, 2002).

The most prominent effects were observed for *Le*-TIMP, *Le*-SOD, *Le*-catalase, *Le*-chitinase and *Le*-HSP70 in the hemocytes of small individuals following acute injury. In the hemocytes of large specimens and siphon tissue of both size groups no strong effects were observed. This emphasizes the role of hemocytes as major players within the immune response in *L. elliptica* and corroborates our earlier findings of higher stress resistance in young compared to old individuals (Husmann et al. 2011; Philipp et al. 2011; Clark et al. submitted). TIMP is an inducible protease inhibitor with a potential function in injury regeneration, remodeling of extracellular matrix and defense reactions. In marine bivalves, TIMP-like molecules have been extensively investigated in the oyster *Crassostrea gigas* where they are most abundant in hemocytes. Their role in the stress response was illustrated by differential expression to bacterial challenge and injury. In *C. gigas* TIMP-like molecules were shown to be up-regulated in response to shell damage or infection with *Vibrio* spp. (Montagnani et al. 2007; Montagnani et al. 2001). However, De Decker and Saulnier (2011) as well as Labreuche et al (2006) found strong down-regulation or no response to *Vibrio* spp. challenge. The discrepancies in expression responses were attributed to the use of different *Vibrio* strains. Given the results presented here, *L. elliptica* *Le*-TIMP, corroborates the role of an important effector in defense mechanisms and wound healing.

The up-regulation of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in injured animals suggested an increased need for cellular protection against increasing oxidative stress levels during acute injury. In bivalves it is known that tissue

lesions or invading bacteria trigger the “oxidative burst”-like generation of cytotoxic ROS including superoxide anions ($O_2^{\cdot-}$) and/or nitric oxides (NO) (Anderson 1994; Bettencourt et al. 2007; Husmann et al. 2011), which are produced during the phagocytosis of particles and invading pathogens (Adema et al. 1991). The observed increase in *Le*-CAT expression indicates enhanced intracellular H_2O_2 generation, which can activate further the immune response pathways involving NF κ B as found in the disk abalone *Haliotis discus discus* (De Zoysa et al. 2009; Ju et al. 2007).

Whereas some Antarctic marine invertebrates such as the sea star *Odontaster validus* or the gammarid *Paraceradocus gibber* lack a classical heat shock response (Clark et al. 2008b; Clark and Peck 2009), experimental work with *L. elliptica* indicated that the HSP70 genes are induced in response to heat stress (Clark et al. 2008a; Park et al. 2007), hypoxia (Clark and Peck 2009; Clark et al. submitted) and also changes in pH (Cummings et al. 2011). Heat shock proteins are often involved in stress defense, and have been shown to be up-regulated in hemocytes upon microbial challenge in bay scallops (*Argopecten irradians*) and zebra mussels (*Dreissena polymorpha*) (Xu and Faisal, 2009; Song et al. (2006). Up-regulation of *Le*-HSP70 in injured *L. elliptica* is further consistent with up-regulation of SOD and catalase, as a response to physiological stress.

The up-regulated expression of *Le*-chitinase in *L. elliptica* following injury is in line with findings in the oyster *C. gigas* (Badariotti et al. 2006; Badariotti et al. 2007a; Badariotti et al. 2007b). mRNA levels of chitinases increased in the hemocytes of *C. gigas* following bacterial challenge and involvement in development, tissue growth and remodeling was reported. In mammals the functions of chitinases include regulation of immunity and apoptosis, stimulation of macrophages, as well as tissue remodeling and wound healing (Lee et al. 2011) and the expression level of chitinase 3-like protein 1 was up-regulated in humans and mice following tissue damage (Bonneh-Barkay et al. 2010; Chen et al. 2004). These data suggest that, similarly, expression of chitinase-like genes in *L. elliptica* hemocytes may be involved in tissue repair or protection from bacterial infection following wound infliction.

The putative novel antimicrobial peptide *Le*-theromacin in *L. elliptica* was identified due to high sequence similarity to the theromacin sequence of the pearl mussel *Hyriopsis cumingii* (Xu et al. 2010). However, its function as an antimicrobial peptide in *L. elliptica* had yet to be confirmed. Expression of *Le*-theromacin was present in all organs of untreated *L. elliptica*, including hemocytes, but no induction upon injury was observed, indicating constitutive expression of this peptide. While *Hc*-theromacin was most strongly expressed in the hemocytes of *H. cumingii* (Xu et al. 2010), theromacin in *L. elliptica* is highly expressed in

fibrous connective tissues such as mantle, foot or siphon compared to hemocytes. This is surprising, since antimicrobial peptides are major humoral effectors in the hemocyte immune response and may be induced by injury or bacterial challenge (Xu et al. 2010). The lack of response in *L. elliptica* may be due to several reasons. Sequence similarity does not necessarily equate to similarity of function (Clark et al. 2002) and whilst *Le*-theromacin and *Hc*-theromacin share high sequence similarity, they may have divergent functions. If however, this is an antimicrobial peptide, then the lack of response could be due to either not sampling at an appropriate time point, or the experimental stimulus was insufficient to induce up-regulation of the gene.

Changes of gene expression in response to siphon injury

Injury to the siphon provoked only minor changes in gene expression in this tissue, although the siphons were visibly affected by the treatment. Older specimens, in particular, were observed to be sluggish, less mobile and less active in filtration when injured. The overall up-regulation of the selected candidate genes in siphon tissue 4 weeks following injury may reflect metabolic long-term changes to the conditions in the holding system without sediment, rather than an explicit response to injury. *Le*-actin expression was up-regulated in the siphons of fed, starved and injured animals after 4 weeks of treatment, possibly indicating investment into tissue maintenance. It is also possible, that the effect of injury on gene transcription is restricted to the injured area alone and the rest of the siphon tissue remains unaffected.

Differences in stress response between younger and older L. elliptica

The most pronounced differences in transcriptional expression were found in the younger animals, indicating that age is a significant factor in the transcriptomic defense response. This substantiates previous work in which hemocyte concentration and the intensity of the oxidative burst response under treatment with bacterial mimetics such as zymosan varied with age. Although small individuals had lower numbers of hemocytes per ml of hemolymph, the ROS generation in response to microbial stimulants was also more intensive compared with older individuals. Experimental treatments such as starvation and acute injury modulated the abundance of hemocytes, especially in older *L. elliptica* (Husmann et al. 2011). The more pronounced changes in gene expression in younger individuals seen in this study as a response to injury is in line with age dependent physiological differences in marine bivalves (Philipp and Abele 2010) and especially in *L. elliptica* (Husmann et al. 2011; Philipp et al. 2005a; Philipp et al. 2005b). Also, the markedly higher susceptibility to injury in older *L.*

elliptica individuals was in line with previous studies, which showed that their mortality rates following injury were higher, their respiration decreased under conditions of high turbidity, and their reburrowing ability was lower in old animals compared to younger specimens (Husmann et al. 2011; Morley et al. 2007; Peck et al. 2004; Philipp et al. 2011). The current gene expression studies investigating the combined effects of starvation and injury now also show that the induction period of stress genes in young animals is shorter compared with old injured animals, indicating a greater resilience and potentially a better physical condition. In summary, these data document the age dependent stress-response capacities in *L. elliptica*. The consequences of such, lead to the prediction that under conditions of environmental stress e.g. ice scour and turbidity from increased glacial run-off, younger animals have a better chance of survival and recovery. This may lead to an alteration in the age structure of the general population towards a higher abundance of young (reproductively immature) animals. On the longer run, this will not only influence the composition and structure of *L. elliptica* populations, but also the composition and function of Antarctic shallow benthic communities (Husmann et al. 2011; Philipp et al. 2011; Siciński et al. 2012).

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Supplementary material

Tab. S1: Effects of injury and feeding conditions on the gene-expression in hemocytes of small and large *L. elliptica* individuals. After laboratory acclimation, individuals were kept under 23 days of “food” and “starvation” conditions and acute (2 days since infliction) injury, as well as 44 days of “food” and “starvation” and recovering from the previous inflicted injury. Data were measured by *real time* PCR and normalized using 18s ribosomal RNA as the reference. Significant effects between groups (N=3-10) and interactions were analyzed by two-way-ANOVA or non-parametric PERMANOVA (“adonis”) and are highlighted by *** (p<0.001), ** (p<0.01) and * (p<0.5). N.s. = not significant.

Treatment	Gene	Small Individuals			Large Individuals		
		Treatment	Time	Interaction	Treatment	Time	Interaction
Injury upon starvation	<i>Le</i> -TIMP	n.s.	*	n.s.	n.s.	n.s.	n.s.
	<i>Le</i> -SOD	n.s.	***	*	n.s.	n.s.	n.s.
	<i>Le</i> -HSP	n.s.	***	*	n.s.	n.s.	n.s.
	<i>Le</i> -Catalase	n.s.	*	*	n.s.	n.s.	n.s.
	<i>Le</i> -Chitinase	n.s.	n.s.	n.s.	n.s.	**	n.s.
	<i>Le</i> -Actin	n.s.	**	n.s.	n.s.	n.s.	n.s.
	<i>Le</i> -Theromacin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Injury upon feeding	<i>Le</i> -TIMP	**	n.s.	*	*	n.s.	n.s.
	<i>Le</i> -SOD	n.d.	n.d.	n.d.	*	n.s.	n.s.
	<i>Le</i> -HSP	***	*	*	n.s.	n.s.	n.s.
	<i>Le</i> -Catalase	**	n.s.	*	n.s.	n.s.	n.s.
	<i>Le</i> -Chitinase	***	n.s.	n.s.	**	n.s.	n.s.
	<i>Le</i> -Actin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	<i>Le</i> -Theromacin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Feeding conditions	<i>Le</i> -TIMP	n.s.	n.s.	**	n.s.	n.s.	n.s.
	<i>Le</i> -SOD	n.s.	n.s.	***	n.s.	*	*
	<i>Le</i> -HSP	n.s.	***	***	n.s.	n.s.	n.s.
	<i>Le</i> -Catalase	*	n.s.	***	n.s.	n.s.	n.s.
	<i>Le</i> -Chitinase	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	<i>Le</i> -Actin	n.s.	*	n.s.	n.s.	n.s.	n.s.
	<i>Le</i> -Theromacin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Tab. S2: Effects of injury and feeding conditions on mRNA expression in the siphon-tissue of small and large *L. elliptica* individuals. After laboratory acclimation, individuals were kept under 23 days of “food” and “starvation” conditions and acute (2 days since infliction) injury, as well as 44 days of “food” and “starvation” and recovering from the previous inflicted injury. Data were measured by *real time* PCR and normalized using 18s ribosomal RNA as the reference. Significant effects between groups (N=3-10) and interactions were analyzed by two-way-ANOVA or non-parametric PERMANOVA (“adonis”) and are highlighted by *** (p<0.001), ** (p<0.01) and * (p<0.5). N.s. = not significant.

Treatment	Gene	Small Individuals			Large Individuals		
		Treatment	Time	Interaction	Treatment	Time	Interaction
Injury upon starvation	<i>Le</i> -TIMP	n.s.	*	n.s.	n.s.	***	n.s.
	<i>Le</i> -SOD	n.s.	**	n.s.	***	**	**
	<i>Le</i> -HSP	n.s.	*	n.s.	n.s.	***	n.s.
	<i>Le</i> -Catalase	n.s.	***	n.s.	n.s.	***	n.s.
	<i>Le</i> -Chitinase	n.s.	*	n.s.	n.s.	**	n.s.
	<i>Le</i> -Actin	n.s.	**	n.s.	n.s.	***	n.s.
	<i>Le</i> -Theromacin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Injury upon feeding	<i>Le</i> -TIMP	n.s.	***	*	n.s.	**	n.s.
	<i>Le</i> -SOD	n.s.	**	n.d.	n.s.	*	n.s.
	<i>Le</i> -HSP	n.s.	n.s.	n.d.	n.s.	***	n.s.
	<i>Le</i> -Catalase	n.s.	***	*	n.s.	**	n.s.
	<i>Le</i> -Chitinase	n.s.	n.s.	n.s.	*	**	n.s.
	<i>Le</i> -Actin	n.s.	*	**	n.s.	**	n.s.
	<i>Le</i> -Theromacin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Feeding conditions	<i>Le</i> -TIMP	n.s.	***	n.s.	n.s.	***	n.s.
	<i>Le</i> -SOD	n.s.	***	n.s.	n.s.	n.s.	n.s.
	<i>Le</i> -HSP	**	**	n.s.	n.s.	***	n.s.
	<i>Le</i> -Catalase	*	***	n.s.	n.s.	***	n.s.
	<i>Le</i> -Chitinase	**	n.s.	n.s.	n.s.	n.s.	n.s.
	<i>Le</i> -Actin	n.s.	***	n.s.	n.s.	***	n.s.
	<i>Le</i> -Theromacin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Tab. S3: *L. elliptica* hemocytes: Expression levels of selected transcripts putatively involved in the stress and immune responses measured by qRT-PCR in small and large *L. elliptica* individuals sampled after 23 days of “feeding” (F) and “starvation” (S) treatment and experiencing acute (2 days after infliction) injury (S/I and F/I). Additional samples were taken after 44 days of “feeding” (F2), “starvation” (S2) and recovering (S/R and F/R) for 21 days from the previous inflicted injury. For experimental details see material and method section. Expression levels were normalized using 18s ribosomal RNA as the reference gene. Data are presented as median. N=3-10 individuals per group.

Treatment "Starvation", young individuals												
Gene	Not Injured _s			Injured _{s/I}			Not Injured _{s2}			Injured _{s/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	2.340	1.328	5.775	1.046	0.109	28.643	0.194	0.143	0.576	0.556	0.252	8.665
SOD	0.065	0.031	0.316	0.025	0.012	0.585	0.003	0.002	0.008	0.017	0.003	0.110
HSP	49.239	9.291	148.646	19.749	2.329	298.800	0.818	0.469	2.664	5.093	1.488	55.680
Catalase	5.390	2.969	17.314	1.779	0.919	23.222	0.398	0.134	2.210	3.333	0.587	19.438
Chitinase	0.280	0.005	0.821	0.053	0.004	0.412	0.052	0.010	0.404	0.073	0.020	3.907
Actin	2320.310	1657.036	4976.668	1960.244	1336.173	13092.320	609.887	333.114	1100.956	1503.006	425.627	6578.380
Theromacin	0.130	0.026	0.259	0.132	0.020	0.486	0.089	0.018	0.402	0.511	0.011	0.732

Treatment "Starvation". old individuals												
Gene	Not Injured _s			Injured _{s/I}			Not Injured _{s2}			Injured _{s/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	3.228	0.329	8.855	1.299	0.257	8.303	0.692	0.089	16.119	4.543	0.373	15.384
SOD	0.098	0.022	0.418	0.016	0.012	0.283	0.013	0.002	0.046	0.024	0.010	0.413
HSP	27.578	5.199	52.247	14.784	4.478	48.974	4.979	0.707	83.089	9.328	3.115	48.358
Catalase	3.463	0.372	12.164	1.250	0.318	6.722	1.258	0.452	13.589	3.342	0.210	37.140
Chitinase	0.009	0.001	0.750	0.006	0.003	0.755	0.164	0.004	0.710	0.174	0.025	1.240
Actin	2582.384	682.788	8912.643	1507.624	500.807	6035.390	1019.639	563.584	7350.837	2951.347	510.965	10769.410
Theromacin	0.012	0.000	0.344	0.050	0.000	0.117	0.009	0.005	0.042	0.071	0.029	0.287

Treatment "Feeding". young individuals												
Gene	Not Injured _F			Injured _{F/I}			Not Injured _{F2}			Injured _{F/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	1.317	0.216	5.800	13.610	4.269	35.904	1.636	1.353	5.405	1.890	1.185	15.423
SOD	0.033	0.014	0.156	0.628	0.091	0.795	0.072	0.026	0.110	0.040	0.021	0.248
HSP	6.430	4.045	132.818	115.114	68.708	353.189	9.671	1.848	53.943	13.438	5.327	57.919
Catalase	2.121	0.807	20.874	38.251	5.665	83.168	5.478	3.363	28.988	6.381	1.474	41.231
Chitinase	0.113	0.031	1.355	1.561	0.097	8.859	0.124	0.116	0.751	1.194	0.163	13.779
Actin	2435.493	398.753	14078.110	3336.244	2059.520	21446.660	2152.994	620.803	4717.120	2702.102	1079.584	7603.034
Theromacin	0.284	0.000	0.948	0.092	0.000	1.450	0.037	0.033	2.469	0.106	0.076	5.970

Treatment "Feeding". old individuals												
Gene	Not Injured _F			Injured _{F/I}			Not Injured _{F2}			Injured _{F/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	0.656	0.029	6.899	2.222	0.598	12.782	3.826	0.149	4.474	4.020	0.323	14.730
SOD	0.020	0.004	0.093	0.087	0.039	0.947	0.037	0.001	0.107	0.039	0.003	0.415
HSP	6.548	0.335	69.176	13.629	1.954	80.411	4.960	0.566	55.211	10.639	2.646	48.819
Catalase	0.458	0.150	8.982	5.078	0.716	28.613	6.593	0.181	8.783	7.002	0.252	20.956
Chitinase	0.008	0.002	0.055	0.093	0.009	2.051	0.036	0.001	0.053	0.413	0.055	0.743
Actin	987.345	74.817	2572.275	2069.699	485.396	4677.746	2347.258	386.409	5258.257	1789.009	258.155	3869.297
Theromacin	0.025	0.009	0.154	0.035	0.000	0.247	0.018	0.000	0.046	0.000	0.000	0.171

Tab. S4: *L. elliptica* siphons: Expression levels of selected transcripts putatively involved in the stress and immune responses measured by qRT-PCR in small and large *L. elliptica* individuals sampled after 23 days of “feeding” (F) and “starvation” (S) treatment and experiencing acute (2 days after infliction) injury (S/I and F/I). Additional samples were taken after 44 days of “feeding” (F2), “starvation” (S2) and recovering (S/R and F/R) for 21 days from the previous inflicted injury. For experimental details see material and method section. Expression levels were normalized using 18s ribosomal RNA as the reference gene. Data are presented as median. N=3-10 individuals per group.

Treatment "Starvation", young individuals												
Gene	Not Injured _s			Injured _{s/I}			Not Injured _{s2}			Injured _{s/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	20.030	14.260	25.670	29.910	12.770	50.630	50.810	17.440	89.140	35.530	15.860	63.630
SOD	8.511	3.739	23.920	17.990	5.274	67.880	46.790	14.680	81.710	47.880	15.050	112.100
HSP	152.800	108.300	184.500	203.100	82.420	421.700	242.300	144.100	485.000	258.100	92.960	326.800
Catalase	123.900	33.240	140.000	106.900	89.670	309.700	368.700	138.200	1177.000	355.000	181.600	481.200
Chitinase	200.800	93.420	742.500	130.800	49.440	501.700	412.000	125.100	1240.000	270.600	178.700	695.100
Actin	213.100	159.100	578.300	302.100	159.800	608.000	531.300	372.300	1361.000	528.100	366.500	745.800
Theromacin	59.120	0.003	106.000	27.720	0.016	59.780	26.140	11.520	230.300	40.030	0.301	202.100

Treatment "Starvation". old individuals												
Gene	Not Injured _s			Injured _{s/I}			Not Injured _{s2}			Injured _{s/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	22.080	10.290	36.090	33.210	14.140	75.340	50.100	30.720	75.340	67.510	24.070	79.250
SOD	15.930	3.331	22.140	19.390	4.255	48.860	11.740	4.063	39.220	58.670	24.320	89.510
Catalase	184.700	85.930	287.800	193.200	81.750	381.200	733.400	365.400	1294.000	894.000	293.300	1321.000
Chitinase	175.500	45.470	261.900	161.800	12.510	416.900	351.000	209.300	467.600	284.300	99.210	417.500
Actin	714.300	320.400	1340.000	820.400	476.400	1534.000	1582.000	809.900	2703.000	2320.000	867.900	2660.000
Theromacin	13.330	0.001	29.070	20.410	0.002	64.450	19.680	0.019	57.150	26.690	0.308	39.410

Treatment "Feeding". young individuals												
Gene	Not Injured _F			Injured _{F/I}			Not Injured _{F2}			Injured _{F/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	15.230	6.771	26.480	28.530	9.100	31.120	33.070	30.070	59.940	27.090	15.780	49.920
SOD	13.900	7.113	34.670	21.690	4.287	44.910	37.280	21.530	69.090	18.690	15.300	72.150
HSP	108.400	48.870	167.400	132.200	45.550	292.800	174.000	99.840	308.500	101.100	70.620	354.500
Catalase	60.010	36.850	108.200	113.000	59.570	171.300	218.500	122.100	321.400	188.800	61.160	402.200
Chitinase	74.540	43.980	362.800	171.700	16.990	529.700	145.100	27.320	273.900	69.640	40.000	396.800
Actin	141.300	53.340	342.100	229.800	114.000	563.700	426.900	288.200	783.200	326.600	164.200	502.900
Theromacin	28.470	0.098	83.750	9.894	0.001	61.950	13.960	5.886	29.580	10.500	0.007	45.510

Treatment "Feeding". old individuals												
Gene	Not Injured _F			Injured _{F/I}			Not Injured _{F2}			Injured _{F/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
TIMP	12.820	4.928	48.040	25.200	1.634	91.780	64.550	27.700	128.800	43.530	11.080	56.190
SOD	7.712	2.078	42.880	13.690	1.262	21.800	25.340	6.475	67.170	10.060	9.859	36.300
Catalase	132.700	44.010	717.700	222.400	10.170	993.000	678.500	358.800	1396.000	407.500	165.500	657.500
Chitinase	49.630	4.580	416.100	38.730	9.225	361.200	421.900	240.800	621.800	221.500	0.213	523.500
Actin	456.700	100.700	1076.000	742.300	47.420	2012.000	2091.000	686.600	3678.000	1595.000	427.400	3000.000
Theromacin	12.820	0.109	36.310	20.350	0.001	53.550	15.500	0.172	119.300	0.676	0.014	10.060

Tab. S5: Effects of injury and feeding conditions on the hepatosomatic index (HSI) of small and large *L. elliptica* individuals. After laboratory acclimation, individuals were kept under 23 days of “food” and “starvation” conditions and acute (2 days since infliction) injury, as well as 44 days of “food” and “starvation” and recovering from the previous inflicted injury. Significant effects between groups (N=5-10) and interactions were analyzed by two-way-ANOVA and are highlighted by ** (p<0.01) and * (p<0.5). N.s. = not significant.

	Small Individuals			Large Individuals		
	Treatment	Time	Interaction	Treatment	Time	Interaction
Feeding conditions	n.s.	n.s.	n.s.	*	n.s.	n.s.
Injury	n.s.	n.s.	n.s.	n.s.	n.s.	**

Tab. S6: Effects of injury and feeding conditions on the hepatosomatic index (HSI) of small and large *L. elliptica*. Individuals sampled after 23 days of “feeding” (F) and “starvation” (S) treatment and experiencing acute (2 days after infliction) injury (S/I and F/I). Additional samples were taken after 44 days of “feeding” (F2), “starvation” (S2) and recovering (S/R and F/R) for 21 days from the previous inflicted injury. For experimental details see materials and methods section. Data are presented as median. N=5-10 individuals per group.

Hepatosomatic index (HSI), young individuals												
Treatment	Not Injured_{S, F}			Injured_{S/I, F/I}			Not Injured_{S2, F2}			Injured_{S/R, F/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
Starvation	7.110	6.268	10.45	8.350	6.683	8.971	8.072	6.584	10.88	7.918	7.446	9.747
Food	8.036	6.762	9.512	8.323	6.258	10.62	8.619	6.961	10.82	8.292	7.623	8.623

Hepatosomatic index (HSI), old individuals												
Treatment	Not Injured_{S, F}			Injured_{S/I, F/I}			Not Injured_{S2, F2}			Injured_{S/R, F/R}		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
Starvation	6.563	5.765	8.451	7.788	7.272	10.79	7.942	6.603	8.891	8.335	6.790	9.176
Food	7.820	7.019	10.62	7.820	6.526	9.159	8.916	7.961	10.12	7.877	6.678	9.070

Tab. S7: Sequence (contig length and number of reads in the RNA sequence database) and protein domain structure of selected *L. elliptica* contigs putatively involved in *L. elliptica* stress response and immune reactions. The protein domain structure in the different contigs was deduced using the NCBI conserved domain architecture retrieval tool cDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) and compared to the conserved domain structure for the respective gene as defined by HomoloGene (<http://www.ncbi.nlm.nih.gov/homologene>). Genes/contigs marked with * were investigated for differential expression in stress treated *L. elliptica* individuals by qRT-PCR. TMH: transmembranhelix. Domains marked with ¹ were deduced from cDART.

Gene name	ENA Accession	Contig length (bp)	Nr. of reads	Domain-containing longest ORF (aa)	Domains expected	Domains identified
Catalase*	HE804714	2439	833	517	Catalase	Catalase_C
Superoxide dismutase Cu-Zn*	HE804717	4964	124	1032	SOD_Cu	5 x SOD_Cu, 2 x TMH
Hydramacin/ Theromacin*	HE804718	655	816	108	None	TMH
Chitinase*	HE804715	3246	255	402	CBM_14, GH18_chitinase-like	GH18_chito, 2x CBM_14
HSP70*	HE804716	2459	528	656	heat shock 70 kDa protein (provisional)	none
Tissue Inhibitor of Metalloproteinase*	HE804719	1924	63	238	NTR_like domain	TIMP
Ferritin*	HE804761	1876	2416	175	Ferritin-like	Euk_Ferritin
Actin*	HE804713	1841	2630	386	Actin	Actin
18s rRNA*	HE804712	2809	5866	173	None	none
AIF	HE804725	1495	171	173	Efh	Efh
Alternative Oxidase	HE804720	1903	19	283	None	AOX, 2 x TMH
Autophagy related Protein 2	HE804753	477	2	147	MRS6, ATG_C	none
Autophagy related Protein 3	HE804754	501	4	135	Autophagy_act_C, ATG_N, ATG_C	ATG_N
Autophagy related Protein 4	HE804756	1734	15	399	Peptidase_C54	Peptidase_C54
Autophagy related Protein 3	HE804755	1011	14	141	Autophagy_act_C, ATG_N, ATG_C	Autophagy_act_C, ATG_C
Autophagy related Protein 4	HE804757	494	3	148	Peptidase_C54	Peptidase_C54
Autophagy related Protein 12	HE804758	1292	16	115	UBQ Ubiquitin-like proteins	APG 12_C
Beta-Glucan Recognition Protein	HE804726	2020	30	543	None	GH16_CCF

Chaperonin	HE804744	872	115	100	Chaperonin_like	Cpn10
Cytochrome p450	HE804759	1884	32	531	Cytochrome P450	P450, TMH
Cytochrome p450	HE804760	1773	24	384	P450	P450
Defensin	HE804727	477	3	66	Defensin propeptide, mammalian defensin	TMH
Dual Oxidase	HE804729	7454	132	917	FRQ1 (Ca2+-binding protein (Efh superfamily)), S-100, An_peroxidase, NADPH_ox, Ferredoxin reductase (FNR)_like, Efh, alpha-dioxygenase, oxidoreductase/ferric-chelate reductase, Ferric_reduct	Ferric_Red, Nox_Duox_1, 2x Efh, 6 x TMH
Dual Oxidase	HE804728	2059	42	101	FRQ1 (Ca2+-binding protein (EF-Hand superfamily)), S-100, An_peroxidase, NADPH_ox, Ferredoxin reductase (FNR)_like, Efh, alpha-dioxygenase, oxidoreductase/ferric-chelate reductase, Ferric_reduct	NAD binding
DnaJ	HE804745	3926	55	210	DnaJ_C, DnaJ	DnaJ_C
Glutathione Peroxidase	HE804721	1387	292	182	Thioredoxin_like	<i>GSH-Peroxi</i>
Glutathione S-Transferase	HE804762	1228	236	227	GST_C_family, Thioredoxin_like	GST_C_Pi, GST_N_Pi
HSP90	HE804748	2834	796	733	HATPase_C, HSP90	2x HSP90, HATPase_C
HSP60	HE804747	3610	68	578	Chaperonin_like	GroEL
HSP, low molecular weight	HE804746	1317	146	165	Alpha-crystallin_HSPs_P23_like	metazoan ACD
Hypoxia Inducible Factor	HE804749	1864	17	493	PAS_3, PAS, HIF-1a_CTAD, HIF-1, HLH (Helix-loop-helix domain)	HLH superfamily, 2 x PAS
Hypoxia Inducible Factor	HE804750	2016	84	338	PAS_3, PAS, HIF-1a_CTAD, HIF-1, HLH (Helix-loop-helix domain)	HIF-1a_CTAD, HIF-1
Interleukin 17	HE804730	560	3	125	IL17	IL17, TMH
MIF	HE804731	705	16	115	MIF	none
Mytilin	HE804732	507	35	94	Myticin-pr ¹	Myticin-pr
Mytilin	HE804733	533	19	102	Myticin-pr ¹	Myticin-pr
Mytimycin	HE804734	395	2	73	None	none
Metallothionein	HE804763	788	659	68	None	none
Nitric Oxide Synthase	HE804736	754	3	251	NOS_oxygenase, FNR_like, FMN_red,	Flavodoxin

					PDZ, NOS oxygenase	
NADPH Oxidase	HE804735	1392	7	202	FNR_like, EFh, Ferric_reduct	Ferric red, FAD-binding, 2x TMH
Nuclear factor NF-kappa-B	HE804737	4499	91	370	Arp, ANK, RHD-n, ankyrin repeat protein, E_set, DD_superfamily, ankyrin-like protein	ANK, Death-NF-kB
Nuclear factor NF-kappa-B	HE804738	2353	36	773	Arp, ANK, RHD-n, ankyrin repeat protein, E_set, DD_superfamily, ankyrin-like protein	RHD-n_NFkB, IPT_Nfkapp, ANK
Peroxiredoxin 5	HE804722	1667	305	204	Thioredoxin_like	PRX5_like
Peroxiredoxin 6	HE804723	2035	110	281	Thioredoxin_like	PRX_1_cys
Prolyl 4-Hydroxylase	HE804751	2317	81	278	2OG-FeII_Oxy, P4Ha_N	P4HC
Peptidoglycan-Recognition Protein	HE804739	1726	123	273	PGRP	PGRP
Superoxide dismutase Mn	HE804724	1261	77	230	SOD_Fe_N, SOD_Fe_C	SOD_Fe_N, SOD_Fe_C
Thioredoxin	HE804752	1445	215	123	Thioredoxin_like	TRX_family
Toll Like Receptor	HE804741	2662	44	713	TIR, TM, LRR	TIR, LRR_RI, COG4886, TMH
Toll Like Receptor	HE804742	1249	12	283	TIR, TM, LRR	TIR, TMH
Toll Like Receptor	HE804743	1742	11	166	TIR, TM, LRR	TIR
TNF receptor associated factor	HE804740	2517	23	541	RING, zf-TRAF, MATH	zf-TRAF, MATH-TRAF3, zf-C3HC4

MANUSCRIPT IV

The impact of sediment deposition and iceberg scour on the Antarctic soft shell clam *Laternula elliptica* at King George Island, Antarctica

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The impact of sediment deposition and iceberg scour on the Antarctic soft shell clam *Laternula elliptica* at King George Island, Antarctica

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Abstract: Recent rapid changes of air temperature on the western side of the Antarctic Peninsula results in increased sediment discharge and ice scouring frequencies in coastal regions. These changes are bound to especially affect slow growing, sessile filter feeders such as the Antarctic bivalve, *Laternula elliptica*, a long-lived and abundant key species with circumpolar distribution. We investigated the effect of sedimentation and ice scouring on small/young and large/old individuals at two closely located stations, distinctly influenced by both types of disturbance. Small individuals dealt better with disturbance in terms of their respiratory response to sediment exposure, reburrowing ability, and survival after injury, compared to larger animals. At the more disturbed station *L. elliptica* population density was lower, but larger animals reburrowed faster after iceberg disturbance and reduced their metabolic rate under strong sediment coverage, compared to larger animals of the less disturbed station, indicating that an adaptation or learning response to both types of disturbance may be possible. Smaller individuals were not influenced. *Laternula elliptica* seems capable of coping with the rapidly changing environmental conditions. Due to a decrease in population density and mean population lifespan, *L. elliptica* could however lose its key role in the benthopelagic carbon flux in areas of high sediment deposition.

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Key words: Antarctic Peninsula, climate change, filter feeders, sedimentation, South Shetland Islands

Introduction

Climate change is often equated with the rise in environmental temperature on regional or global scales and indeed, global air temperature has risen by about $0.6 \pm 0.2^\circ\text{C}$ over the past century. The western Antarctic Peninsula (WAP) is one of the most rapidly warming regions on Earth (Vaughan *et al.* 2003, Turner *et al.* 2005). Here most recent reconstructions show air temperatures rose by $0.11 \pm 0.04^\circ\text{C}$ per decade between 1957 and 2006, which amounts to a total maximum rise of 0.55°C within the 50 years of measurements (Steig *et al.* 2009). Effects of local air warming at the WAP meanwhile transfer to the marine environment with increasing water temperatures (Meredith & King 2006, Whitehouse *et al.* 2008), and major changes in coastal and shelf pelagic systems have already become apparent (Ducklow *et al.* 2007). It is projected that changes will continue if the current warming trend proceeds (Dierssen *et al.* 2002, Moline *et al.* 2004).

A major factor of disturbance on the shallow Antarctic shelf is through the impact of iceberg scouring, foot-, anchor-, or fast ice on the benthic fauna (Barnes & Conlan 2007). As glaciers disintegrate due to increasing air temperatures, the number of icebergs is predicted to

increase in the coastal area of the WAP (Barnes & Conlan 2007, Smale & Barnes 2008), although long-term recordings are still missing. Icebergs beaching in shallow coastal areas cause disturbances of the coastal benthic fauna by ice scouring and are predicted to affect bottom community structure and biodiversity on ecological timescales (Smale & Barnes 2008). Site-specific investigations of ice scouring frequency and depth profiles in the WAP region in recent years indicate that scour frequency decreases with depth (Barnes 1999 for Signy Island, South Orkney Islands; Brown *et al.* 2004 & Smale *et al.* 2007 for Adelaide Island, WAP). Further, Smale and Barnes (2008) found ice scour frequency at a given site to be most clearly dependent on the duration of winter fast ice, which immobilizes icebergs in shallow areas and reduces ice scouring. Future predictions of a late build-up and short duration of winter fast ice cover (Vaughan *et al.* 2003) would consequently increase local scouring intensity.

The immediate effect of ice scour on bottom communities is extraordinary. Around Adelaide Island, Smale *et al.* (2007) reported a > 90% reduction in species abundance and a > 60% reduction in species-richness and Peck *et al.* (1999) reported even a > 99% reduction in macrofauna abundance by iceberg impact on a soft sediment habitat at Signy Island.

Sessile species such as bryozoans are more heavily impacted by ice scour than mobile species such as sea urchins (Brown *et al.* 2004) which are able to evade the icebergs' movements. As iceberg scouring inflicts damage on sessile bottom fauna and exposes infaunal species on the sediment surface they support populations of mobile predators such as sea-stars and fishes (Barnes & Conlan 2007, but see Smale *et al.* 2007). After removal of *Laternula elliptica* (King & Broderip) by divers from the sediment at South Bay, Palmer Archipelago (WAP), only 60% of specimens were able to rebury fast enough to avoid predation by the sea-star *Odontaster validus* (Koehler) and the nemertean *Parborlasia corrugatus* (McIntosh) (Zamorano *et al.* 1986).

Higher air temperatures result in longer summer melt periods, higher melting rates and larger areas affected by melting in the northern WAP region (Rau & Braun 2002, Vaughan 2006, Dominguez & Eraso 2007). Freshwater discharge into the ocean and the consequent drop in salinity can affect nearshore and offshore (> 100 km) phytoplankton blooms (Dierssen *et al.* 2002). Together with the high load of inorganic particles such discharge can further impact benthic suspension feeders, as shown for temperate bivalve species. Madon *et al.* (1998) for example, reported decreased clearance, ingestion and absorbance rates as well as food assimilation efficiencies at increasing concentrations of inorganic particles in the water, leading to a decline in food quality and quantity available for North American zebra mussels, *Dreissena polymorpha* (Pallas).

In the scallop *Pecten novaezelandiae* Reeve from New Zealand lower ciliary activity on the bivalve gill surfaces was measured when covered with sedimentary matter, and also a higher animal mortality occurred as silt concentrations increased (Stevens 1987).

The bivalve *Laternula elliptica* of the present study is an infaunal key species in Antarctic coastal environments (Momo *et al.* 2002) and often forms the major biomass component in the bottom communities with densities of > 250 individuals m⁻² (De Laca & Lipps 1976, Ahn 1994, Urban & Mercuri 1998, present study). We hypothesize that changes in disturbance frequency by ice scours and alterations in particle sedimentation in systems fronting melting glaciers will affect *L. elliptica* physiology and alter the population structure. Changes of filtration and bio-deposition by this key species are bound to alter energy flux in the sediment–water interface and thereby the energy supply to benthic communities (Momo *et al.* 2002).

In the present study the effects of sedimentation and ice scouring were investigated in a set of field and laboratory experiments at Potter Cove, King George Island. Glacial meltwater discharge and the plumes of terrigenous sediment are a well-known phenomenon in Potter Cove (Klöser *et al.* 1994, Varela 1998). Individuals from a location already experiencing high sediment impact and ice scouring frequencies within the runoff plume of Collins Glacier were compared to individuals collected from a station more remote from the glacial effluents and less

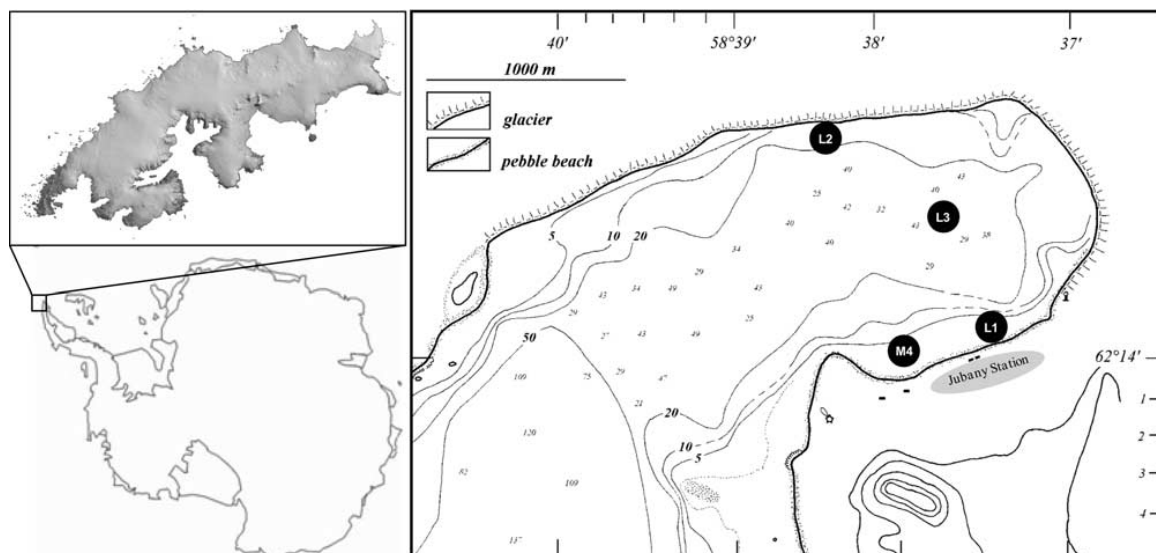


Fig. 1. Maps of the location of King George Island (KGI) on the western Antarctic Peninsula (boxed), Potter Cove on KGI (boxed) and Potter Cove with depth profile. Dots mark the two sampling sites of *Laternula elliptica*. 1) L1 = disturbed population, 2) L2 = undisturbed population, 3) L3 = station for water samples, and 4) M4 = disturbed station, only used for abundance measurements. Position of the Argentinean Jubany Station is given. KGI map was taken from www.kgis.scar.org (accessed July 2010, now available on <http://www.geographie.uni-freiburg.de/ipg/forschung/ap3/kgis/>). Potter Cove map was modified after an original drawing by Grita Veit-Koehler, DZMB, Wilhelmshaven.

affected by ice scour. Experiments were carried out in a size-dependent manner, to check for size/age effects in animal susceptibility to both forms of impact. This was done to predict the consequence of disturbance at the population level, but also to see whether *L. elliptica* is able to adapt its behaviour and physiology under both climate change induced disturbance effects.

Material and methods

Laternula elliptica for the laboratory experiments were collected by divers between November 2006 and February 2007 at two stations in Potter Cove, King George Island, South Shetland Islands. Station L1 (Fig. 1; 62°14'15.0 S, 058°39'48.0 W) with an observed high frequency of ice scouring is situated within the sediment runoff plume of Collins Glacier close to Jubany Station, and animals were sampled at around 7 m depth. Due to the current system in Potter Cove (Klöser *et al.* 1994), icebergs are often found beached in front of Jubany Station and impact L1, whereas station L2 (Fig. 1; 62°13'51.1 S, 058°39'57.5 W) is situated on the opposite coastline of Potter Cove and icebergs are rarely seen in that area. Animals at L2 were sampled at around 15 m depth. At the Dallmann Laboratory, Jubany Station, bivalves were kept in a constant temperature room at 1°C with running natural seawater from the cove. The injury experiment was undertaken in the field season 2008–09. Animals were collected at the more impacted L1 station and kept in the laboratory as described above.

Water samples for the assessment of sediment concentration were taken at L1 and L2. For a better overview of the transition zone of the sediment plume, additional water samples were taken at a station between the two sampling stations (L3; Fig. 1; 62°13'57.4 S, 058°39'56.0 W). A fourth station M4 (Fig. 1; 62°14'21.4 S, 058°40'26.0 W) fronting a meltwater creek outlet ('Matias creek', unofficial name) and similarly impacted by sedimentation and iceberg scour as L1 was included only for the assessment of iceberg effects on *L. elliptica* abundance, additionally to L1 and L2.

Sediment concentrations in the field

To investigate the differences in total suspended particulate matter (SPM) between the L1 and L2 stations during the summer season, SPM concentrations were measured at weekly intervals in different water depths. Water samples were taken using a 5 l Niskin bottle in 0 and 5 m water depth and close to the sea floor (7–10 m at L1 and 13–18 m at L2). Additionally, station L3 was sampled at 0, 10, 15 m, and at 30 m water depth, which is close to the bottom. Two parallel samples were taken at each water depth. Depending on the sediment load, 250–2000 ml of water were filtered over pre-weighed cellulose-acetate filters (142 mm diameter, 0.2 µm pore size, Sartorius AG,

Germany) and dried for at least three days at 60°C to determine dry weight. Subsequently, filters were combusted at 500°C for five hours to determine percentage organic matter (OM). Additionally, secchi depths were recorded at each sediment sampling event at the different stations.

Laternula elliptica natural density at different in situ stations

The *in situ* density of individuals per square metre was investigated at stations L1, L2, and M4. At each station 274–377 underwater pictures covering 0.081 m² of sediment surface, each, were taken by divers using an underwater camera (Nikon Coolpix 9000 in a Sealux housing with Nikon Flash SB103). Bivalve densities were estimated by counts of visible siphons on the sea floor and calculated as mean density per square metre at the respective station.

Respiration at different sediment concentrations

Laternula elliptica of differing size between 35.49 and 91.47 mm shell lengths were sampled at stations L1 and L2. Prior to the measurements, bivalves were maintained without food for three days to minimize as much as possible the effect of specific dynamic action without starving the animals. Standard aerobic metabolic rate was recorded in a multi-channel intermittent flow-through system with oxygen sensors spots placed onto the chamber wall and recording done from the outside using glass fibre oxygen optodes and a Fibox 4-channel system (PreSens® GmbH). The respiration chambers were perspex cylinders of different volumes, adjustable to the size of the animals (550–1650 ml). Water flow was maintained by pumps connected to the respiration chambers with gas-tight tubes and creating a forward flow of c. 3 ml min⁻¹. Experimental conditions were maintained at 0 ± 0.4°C and 34 PSU. Non-invasive oxygen sensors were calibrated to 100% oxygen solubility in air-saturated and to 0% in N₂-saturated seawater at experimental temperature.

Bivalves were allowed to accommodate to the respiration chambers overnight, which were located in a 100 l aquarium in which constant 100% air saturation was achieved by air pumps. A measurement cycle was initiated by switching the system from 'flow-through' to 'closed-circuit flow' status. The water in the closed system was continuously circulated by separate pumps, and the decrease in the oxygen content from 100% to c. 70% oxygen was recorded over time. When reaching 70% oxygen saturation, the system was switched back to the 'flow-through' mode until 100% saturation was again reached, whereupon a new run was started by closing the chambers. One to two runs of four to five hours each were carried out for each animal. Initial respiration measurements were undertaken with natural seawater without sediment as controls. For the sediment treatments, 300 and 600 mg of natural sediment per litre of seawater were added stepwise to the 100 l aquarium, housing the respiration

chambers, 12 hours before the respiration measurement was started by switching to 'closed-circuit flow' status. Sediment for the experiments was taken from the runoff of the glacier at the very end of Potter Cove, where fine sediment had dried to plaques on the side of the riverbeds. The sediment was ground in a mortar and sieved through a 50 μm mesh size sediment sieve. Sediment organic content was determined gravimetrically (see above for field measurements). To achieve a constant 5% of organic matter, the algae *Desmarestia antarctica* (Moe & Silva) was sampled, dried, ground to fine powder and added to the sediment. Over the course of the experiment, the sediment could not be maintained in complete suspension and sedimentation occurred in the experimental tanks as well as inside the respiration chambers, although we increased the water flow velocity in the holding tank. In spite of these difficulties, suspended sediment concentrations of $54 \pm 2.8 \text{ mg l}^{-1}$ in the 300 mg l^{-1} experiment, and $76.4 \pm 1.13 \text{ mg l}^{-1}$ in the 600 mg l^{-1} experiment, could be maintained for a period of > 24 hours, gravimetrically controlled as described above. The setup mimicked the conditions in the field, in which warm days will lead to a peak in glacial sediment runoff which slowly decrease again when discharge rates are lower on cold days (Dominguez & Eraso 2007).

Respiration measurements with each animal lasted three days, starting with an acclimation phase overnight and respiration measurements without sediment the next day. In the evening 300 mg l^{-1} of sediment were added and after 12 hours exposure, respiration was recorded over the next day. On the next evening, 600 mg l^{-1} of sediment was added and the measurement continued throughout another day. Immediately upon completion of the last measurement cycle, the animals were dissected and soft tissue wet mass determined. The soft tissue was dried at 60°C for at least three days to determine dry mass. Standard metabolic rate (SMR) was calculated after subtraction of the microbial oxygen demand determined in a parallel blank chamber. Percentage O_2 saturation was transformed to micromoles of dissolved oxygen in seawater using values of oxygen solubility obtained from Benson & Krause (1984).

Animals displayed frequent respiration pauses lasting up to 30 minutes, in the following called interrupted respiration patterns (IPs), and already described for *L. elliptica* by Morley *et al.* (2007). Occurrence and duration (min) of IPs were recorded and compared between treatments (sediment concentration) and sampling location (L1 or L2).

Burrowing experiments in the field

A steel frame with an inner area of 0.5 m^2 was placed on the surface at stations L1 and L2. Each frame position was marked at the surface with an attached buoy. Four experiments were conducted at each station involving 9–18 *L. elliptica* individuals of different sizes (small,

medium, large). The animals were dug from the sediment by divers and deposited on top of the enclosed sediment area. Pictures were taken directly after exposing the animals in the frame and thereafter at *c.* 24 hour intervals, depending on the weather conditions, for up to 107 hours. As we observed that smaller animals reburied very fast, one experiment exclusively with small-sized animals from L1 and L2 was undertaken at station L1, to see whether smaller animals from the two sites burrowed at the same speed into the same sediment. Eleven animals of each station were deposited on the two sides of the steel frame and photos were taken at 0, 3 and 6 hours following exposure. Following each experiment, animals were taken to the laboratory for morphological measurements (size, weight).

Unfortunately shells from all experiments above (respiration, burrowing) were lost in a ship fire on the Argentine ice breaker ARA *Almirante Irizar*, so that animal age could not be determined by shell ring counts and results can only be related to size but not to age.

Injury experiment: simulation of ice scouring in the laboratory

Ice scouring events will not only expose *L. elliptica* to predators by ploughing them out of the ground. The animals can be injured by the crushing force of the ice (supplementary data will be found at www.journals.cambridge.org/jid_ANS). In an injury experiment, the susceptibility of different *L. elliptica* size/age classes to different forms of injury was investigated. Small (33–40 mm) and large (70–85 mm) animals of station L1 were injured either by applying two cuts in the siphon, mantle and foot muscle or by cracking the shell by a soft blow with a blunt tool (wrench) (Ziuganov *et al.* 2000). Each type of injury was separately inflicted to a total number of 12 animals of each size class. Control animals were not injured. The animals were kept in four large aquaria (180 l) with the different sizes and injuries mixed. The experiment was terminated after 38 days when all animals of two injury groups had died.

Statistical analysis

Statistical analysis was carried out using Graph Pad software (version 5.01). Data were tested for Gaussian distribution and variance homogeneity. Slopes of the respiration experiments of differently sized individuals from the two stations and under different sediment treatments were compared using linear regression analysis and slopes and intercepts analysed for significant differences (ANCOVA). Determination of significant differences in respiration response between different animal sizes was undertaken with the Johnson-Neyman technique (Johnson & Neyman 1936), using a programme provided by C. White (White 2003). Data for suspended sediments were compared

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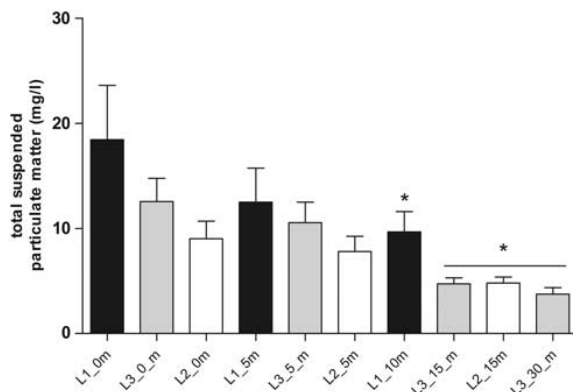


Fig. 2. Field measurements of sediment load in different water depth of the disturbed (L1) and undisturbed (L2) sampling station of *L. elliptica* and an in-between (L3) station. Shown are means \pm standard error of the mean (sem) of total suspended particulate matter (SPM, inorganic and organic) of 11 weekly measurements per depth, starting on 27 December 2006 (sampling event 1) and ending on 9 March 2007 (sampling event 11). Bottom measurements of the stations L2 (15 m) and L3 (30 m and 15 m water depth) were different from L1 (10 m) with $P < 0.05$ (ANOVA).

between stations and depths, and IP frequencies and durations were analysed using *t*-test. Differences between three and more groups were analysed with ANOVA (Kruskal Wallis in case of non-Gaussian distribution) and the Tukey Post-hoc Test (Dunn's Multiple Comparison in case of non-Gaussian distribution). Survival curves of the injury experiment were analysed using the survival curve analysis (Kaplan-Meyer) of Graph Pad Prism software 5.01.

Results

Suspended particulate matter (organic and inorganic) concentrations in Potter Cove

The concentration of suspended particulate matter (organic + inorganic) in Potter Cove was highly variable in summer 2007. In surface water at station L1 values ranged from 2.6–57.73 mg l⁻¹, at station L3 from 3.05–24 mg l⁻¹ and at station L2 from 1.8–22.8 mg l⁻¹. Close to the bottom concentrations were 2.21–20 mg l⁻¹ at L1 (10 m), 1.27–7.38 mg l⁻¹ at L3 (30 m) and 1.4–7.9 mg l⁻¹ at L2 (15 m). In general, *in situ* SPM concentrations followed the expected gradient across Potter Cove with L1 > L3 > L2 (Fig. 2). Decreasing concentrations were observed at greater depth (Fig. 2) as the sediment runoff plume is mostly confined to the upper 0–5 m layer of the water column. Due to the high temporal variability during the field season a significant difference in SPM concentration between stations L2 and L1 with L3 was observed only in the bottom layer (Fig. 2). However, the

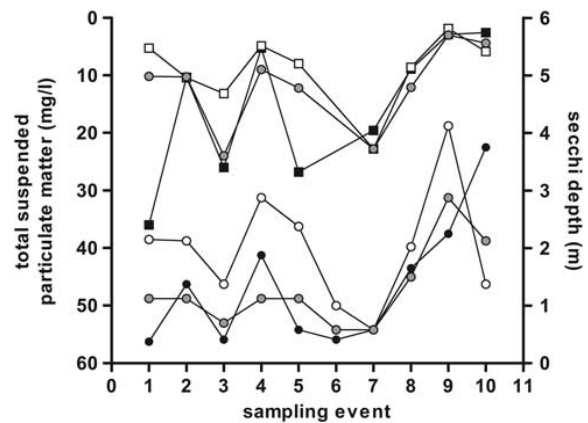


Fig. 3. Weekly measurements of secchi depth (circles) and gravimetrically analysed surface water SPM (squares) throughout the season (27 December 2006–9 March 2007) at the stations L1 (black symbols), L2 (white symbols) and L3 (grey symbols). $n =$ two per sampling event.

results corroborate the initial assumption that animals from L1, which are closer to the glacier and downstream of the glacier outflow, are exposed to higher sedimentation rates than animals from L2 (1 km away from the glacier and upstream of the outflow). Parallel measurements of secchi depth indicate that SPM concentrations higher than 10 mg l⁻¹ can be reliably estimated by secchi depth measurements,

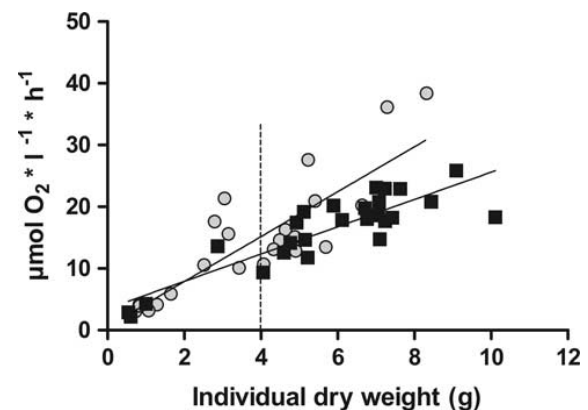


Fig. 4. Respiration rate of L1 (grey circles) and L2 (black squares) individuals under control condition (no sediment). Each data point represents the mean of two measurements for one individual. Size range for L1 is 39.83–84.02 mm, and for L2 is 35.49–91.47 mm. Slopes are significantly different with $F = 7.39$, degrees of freedom numerator (dfn) = 1, degrees of freedom denominator (dfd) = 46, $P = 0.0092$. $n = 24$ (L1), 25 (L2). Broken line = region of no significance (min: -2.239, max: 4.04 gDW), calculated using Johnson-Neyman technique Scatter Plot (White 2003).

whereas for lower concentrations this method is not sensitive enough and also the secchi disk is not applicable in greater water depths. Nevertheless, the secchi measurements also indicate station L2 to be less influenced by sediment (higher secchi depth) than stations L1 and L3 (Fig. 3). Stations L1 and L3 were very similar with respect to secchi depths, indicating similar sediment conditions in both locations.

Laternula elliptica densities at the experimental stations in Potter Cove

The analysis of the underwater pictures from the disturbed station L1, the 'Matias creek' Station M4, and the undisturbed station L2 revealed *c.* 30% higher mean

individual density of *L. elliptica* at L2 (34.20 ± 39.54 SD individuals m^{-2} , number of analysed pictures = 274) compared to L1 (23.78 ± 28.78 SD individuals m^{-2} , number of analysed pictures = 377) and M4 (25.31 ± 35.23 SD individuals m^{-2} , number of analysed pictures = 278). Stations L1 and M4 were not significantly different (ANOVA) and both were less densely colonized than the undisturbed L2 site.

Respiration measurements

When comparing *L. elliptica* from the disturbed L1 and the undisturbed L2 station that had not been experimentally treated with sediment, we found that standard metabolic rates increased more clearly with animal size (age) in animals from L1 compared to the undisturbed station (L2, Fig. 4). Using the Johnson-Neyman technique, significantly higher whole animal respiration at L1 compared to L2 was recorded only in individuals heavier than 4 g tissue dry mass (Fig. 4).

Treatment of experimental animals with 12 hours of 54 and 76 mg sediment l^{-1} had different effects on the respiration rate of animals from both stations (Fig. 5). The respiration rates of the animals from the undisturbed L2 station were not affected by either of the two sediment

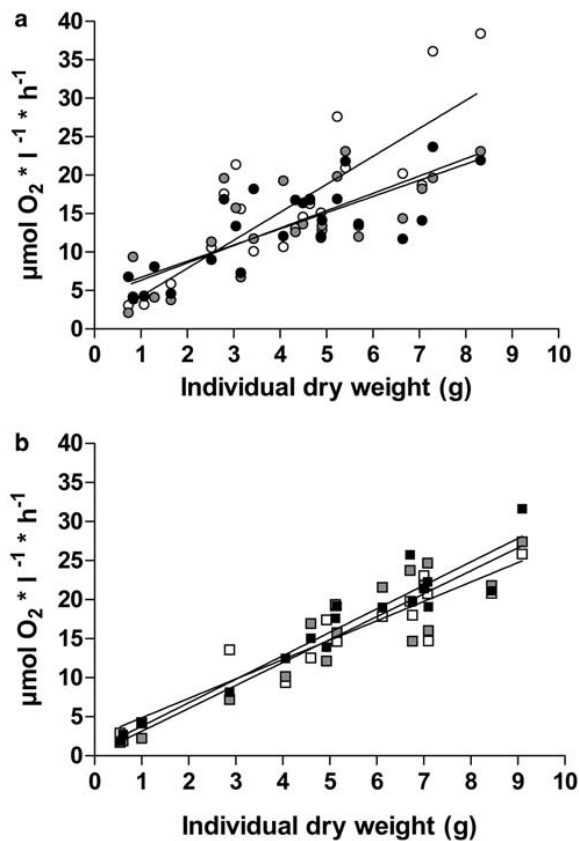


Fig. 5. Respiration rates ($\mu\text{mol O}_2 \text{ l}^{-1} \text{ h}^{-1}$) plotted against dry mass (g) of **a.** L1 (circles), and **b.** L2 (squares) animals under control conditions (clear symbols, no sediment) or 54 ± 2.8 mg sediment l^{-1} (grey symbols) and 76.4 ± 1.13 mg sediment l^{-1} (black symbols) incubation. Slopes were not significantly different for L2 animals. Slope of control animals from the L1 station were significantly different from 54 ± 2.8 mg l^{-1} ($F = 4.95$, $dfn = 1$, $dfd = 44$, $P = 0.031$) and 76.4 ± 1.13 mg l^{-1} ($F = 6.53$, $dfn = 1$, $dfd = 44$, $P = 0.014$) treated animals. Both sediment treatments of L1 animals were not significantly different. $n = 24$ (L1), 17 (L2).

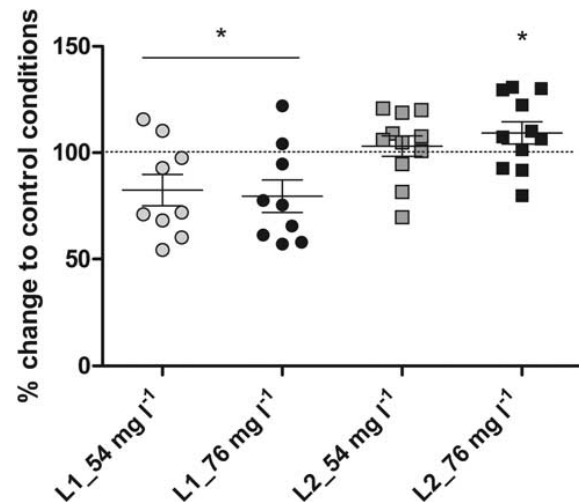


Fig. 6. Percentage change in respiration compared to control conditions without sediment of animals from stations L1 (circles) and L2 (squares) under 54 ± 2.8 mg sediment l^{-1} (grey symbols) and 76.4 ± 1.13 mg sediment l^{-1} (black symbols) in the incubation water. Each data point represents the mean of two replicate measurements with the same individual. L1: range of dry weight = 4.88–8.32 g, mean 6.16 ± 1.22 SD ($n = 9$), L2: range of dry weight = 4.9–9.1 g, mean 6.68 ± 1.3 SD ($n = 11$). Dry weight of experimental animals did not differ significantly between both experimental groups from the stations. * = significant difference between groups with $P < 0.05$. Dotted line marks 100% control respiration without sediment treatment.

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Table I. Analysis of interrupted respiration patterns (IPs). For each individual of the different stations and sediment treatments the occurrence of apneal patterns was recorded.

Station	Sediment (mg l ⁻¹)	n (total)	Occurrence of IP			Duration of IP				
			No. of animals with IPs	No. of animals without IPs	% animals with IPs	n (with IPs)	Mean duration respiring (min)	SD	Mean duration interrupted (min)	SD
L1	0	24	15	9	63	15	0.83	0.41	0.87	0.27
L1	54	24	12	12	50	12	0.79	0.40	0.86	0.28
L1	76	24	16	8	67	16	0.81	0.37	0.90	0.32
L2	0	17	16	1	94	16	0.83	0.39	1.04	0.59
L2	54	17	12	5	71	10	1.03	0.24	1.00	0.61
L2	76	17	13	4	76	13	0.90	0.25	0.86	0.29

concentrations (Fig. 5b), although these concentrations are well beyond the values the animals experience in their natural environment (see above field sedimentation measurements). In contrast, larger animals from the disturbed L1 station reduced respiration rates by on average 20% when exposed to either sediment concentration, compared to non-treated control animals. This response did not differ between different amounts of sediment in large L1 specimens (Fig. 5a). Using the Johnson-Neyman technique, a comparison between respiration rates of L1 animals under control conditions and exposed to the highest sediment concentration (76 mg sediment l⁻¹) showed that animals of > 4.68 g tissue dry mass respired significantly less under sediment treatment compared to sediment free control conditions ($P < 0.05$) whereas respiration rates of animals of < 4.68 g tissue weight was not affected by sediment exposure. Comparing respiration rates of control animals and animals kept with 54 mg l⁻¹ sediment, no animal size-region of non-significance could be defined with a $P < 0.05$ although slopes were significantly different ($P < 0.05$). When setting the significance level to $P < 0.1$, again animals of > 4.56 g tissue dry mass had significantly lower respiration rates under sediment treatment compared to control conditions.

For a better quantification of sediment effects on respiration, the response was calculated for individual specimens from stations L1 and L2 in percent increase or decrease compared to the control rates (without sediment) of the same animal. ANOVA of the entire set of small and large animals studied at both stations found no significant difference in the quantitative response of L1 and L2 animals to either sediment concentration. However, when only animals beyond the limit defined by the Johnson-Neyman technique for the L1 station (> 4.68 g dry mass) were compared, again large L1 animals displayed a significant decrease of respiration in both sediment treatments, whereas L2 animal respiration even increased slightly (average < 10%) above the 100% line of the control rates (Fig. 6).

A higher percentage of animals from the L2 station displayed IPs compared to animals from the L1 station (Table I) in all three treatments. In animals of both stations which showed IPs, the pattern did not differ between control and sediment treatments. Also, IP duration, i.e. the time individuals spent respiring or not, did not vary between stations or treatments. Instead, all L1 and L2 animals which were showing IPs spent a similar time in the active or interrupted respiration phase (Table I).

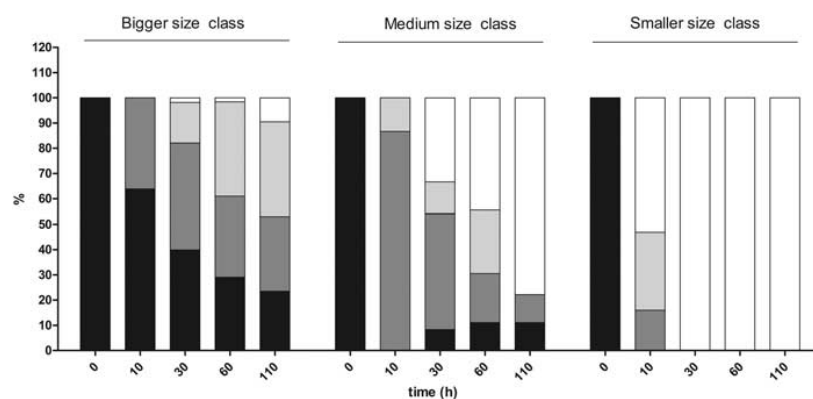


Fig. 7. Velocity of reburrowing in different *L. elliptica* size classes, both stations combined. Differently shaded bars represent mean percent of *L. elliptica* remaining at the sediment surface (black), 50% reburrowed (grey), 75% reburrowed (light grey) or completely reburrowed (white) after 0, 10, 30, 60 and 110 hours in the different size classes. $n = 8$ experiments (53 animals) for large size class (shell length 76.04 mean \pm 0.86 sem), 4 experiments (14 animals) for medium size class (shell length 62.88 mean \pm 1.68 sem) and 5 experiments (60 animals) for smaller size group (shell length 37.31 mean \pm 0.56 sem).

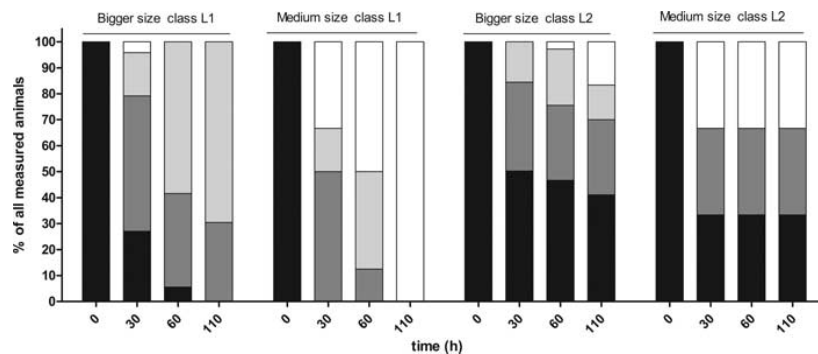


Fig. 8. Burrowing experiment separated in stations. Differently shaded bars represent mean percent of animals which lay on top of the sediment (black) or were 50% (grey), 75% (light grey) or completely reburrowed (white) after 0, 30, 60 and 110 hours in the different size classes. L1: $n = 4$ experiments (22 animals) for large size class ($75.84 \text{ mean} \pm 1.03 \text{ sem}$), 3 experiments (11 animals) for medium size class (62.07 ± 1.96); L2: $n = 4$ experiments (31 animals) for large size class (76.18 ± 1.282), 1 experiment (3 animals) for medium size class (65.81 ± 3.14).

Burrowing experiments in the field

Smaller animals reburrowed faster than medium- and large-sized animals (Fig. 7). Between stations, no difference was observed between small individuals from stations L1 and L2. In a short-term experiment ten out of eleven small animals of both stations were already completely burrowed at the first time point, i.e. after three hours, and all small animals had reburrowed after six hours. When only large- and medium-sized animals are considered, individuals

from the disturbed L1 station appeared to burrow faster compared to animals from station L2 (Fig. 8).

Injury experiment: simulation of ice scouring in the laboratory

Neither in the injury groups nor in the control group did any of the small individuals die during the 38 days experimental period. Within the large animal group, only one out of 12 animals died in the control group at day 38. In the injury groups, animals started to die at day 11 and continued to die so that on day 38 none of the animals inflicted with shell cracks and muscle cuts were alive. In the group of animals with siphon cuts only two animals out of twelve were still alive after 38 days (Fig. 9).

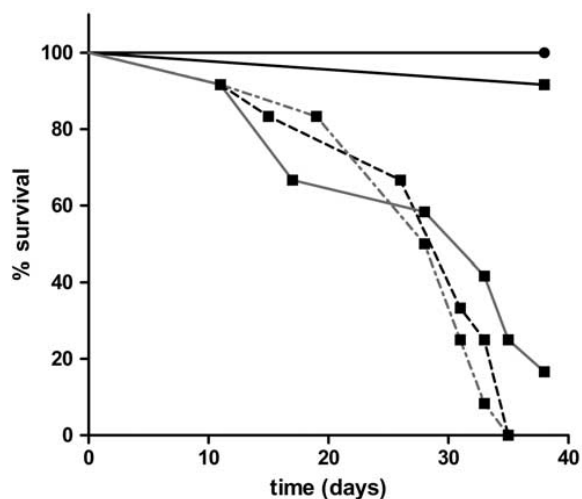


Fig. 9. Percentage survival of animals under simulated ice scouring inflicted injury experiment in the laboratory. Small (circles) and large animals (squares) were subjected to siphon cuts (grey line), adductor muscle cuts (black broken line) and shell cracks (grey broken line) as well as without injuries as control (black solid line). Note that all lines of the small animal groups are overlapping as no animal died during the treatment.

Discussion

The shallow Antarctic shelf areas off the WAP, where the animals of this study were sampled, is already influenced by increasing air and water temperatures, and future climate change scenarios predict continued aerial and ocean warming (Meredith & King 2006, Ducklow *et al.* 2007, Smale & Barnes 2008). The shallow areas in Potter Cove are periodically smothered by sediment runoff and abraded by icebergs, one of the most intense natural forces in Antarctic shallow water environments (Gutt 2001, Barnes *et al.* 2006). In the present study we showed that *L. elliptica* experiences different sediment loads in Potter Cove depending on the location and the distance from the glacial runoff source, wind speed and direction, and the depth distribution of the animals. At station L1, which was situated within the plume of glacial runoff, highest values between 19 and 36 mg l^{-1} SPM were recorded at 5 and 10 m water depth, where a high number of *L. elliptica* were found. On the opposite side of the cove, animals were less

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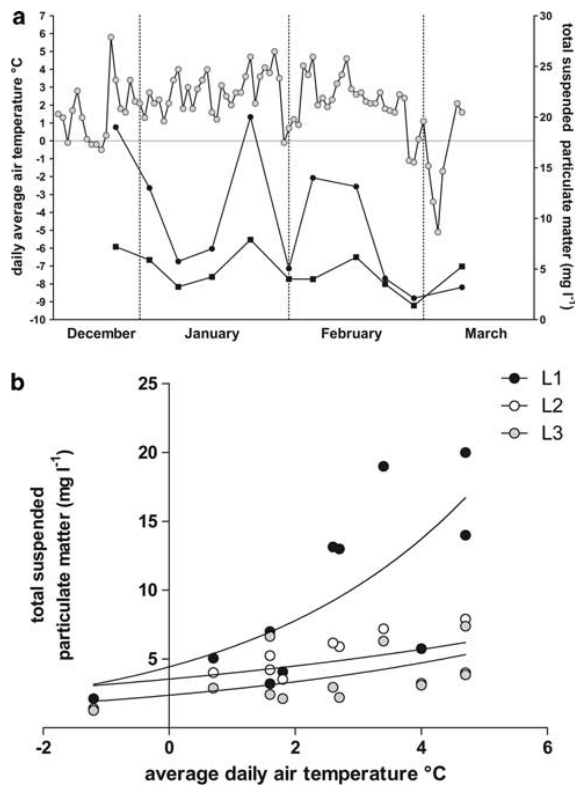


Fig. 10. a. Individual weekly measurements of SPM in bottom water of the undisturbed station (L2, squares) and the disturbed station (L1, circles) as well as daily average air temperatures (grey circles). Note the high variance in sediment load during the season which follows the air temperature pattern. b. Bottom measurements of SPM on the three sampling stations plotted against the daily average air temperatures at the specific sampling date. Sampling time from 27 December 2006–9 March 2007. Air temperature data from www.tutiempo.net/en/Climate/BASE_JUBANY/10-2001/890530.htm, accessed December 2009).

affected by sedimentation and peak values at the bottom (15 m) amounted only to 7.9 mg l^{-1} SPM (for comparison with L1: 20 mg l^{-1} at 5 m depth). Even our small dataset of SPM in the water column indicates that the daily air temperatures seem to have an immediate influence on sediment discharge into the cove. Peaks of SPM at bottom depths were observed mostly following a short period of rapid warming of air temperatures (Fig. 10a & b) and SPM values correlate well with the daily fluctuating air temperature maxima (Fig. 10b). This is in keeping with Dominguez & Eraso (2007) who reported a direct effect of air temperature on sediment discharge at Collins Glacier for the Potter Cove region. Our data document the immediate effect of short-term rises of air temperature on the marine communities in the vicinity of the glaciers.

When temperatures drop at the end of summer (March), creeks freeze and sediment discharge comes to a halt.

Both intense sedimentation and ice scouring impact *L. elliptica* physiology and population dynamics. In our study we analysed several features of bivalve behaviour under stress exposure in relation to size/age of the animals from stations L1 and L2 and some differences emerged. Larger individuals from strongly affected L1 changed respiration rates in response to sediment treatment whereas small animals did not, and on both stations larger animals reburrowed less rapidly and were much more susceptible to injury compared to small individuals. Concerted sedimentation and iceberg stress in an area like Potter Cove could therefore lead to a shift in the population size/age structure of *L. elliptica* towards more smaller/younger individuals. This is in line with observations of Peck & Bullough (1993) and Brown *et al.* (2004) who found a shift towards smaller/younger individuals for the bivalve *Yoldia eightsi* (Couthouy) and the bryozoans *Fenestrulina rugula* (Hayward & Ryland) in areas around Signy and Adelaide Island with high ice scouring frequency, compared to less disturbed sites.

However, the respiratory decrease under sedimentation and faster reburrowing response in the larger individuals of the disturbed compared to the less disturbed station may be first indications of an adaptation the animals undergo when exposed to increased sediment and iceberg impact. Fast reburrowing is necessary in areas with high ice scouring frequency which unearth the individuals from the sediment and expose them to predators. Decreasing respiration rates during sediment exposure has been described for several bivalve species (see Summers *et al.* 1996) but the mechanism causing the reduction is not clear. Interrupted respiration event patterns recorded in the present study indicate *L. elliptica* do not close siphons more often under sediment treatment than under control conditions. Hence, the decreasing respiration rates might be due to impairment of ciliary activity by the silt film covering the gill surface (Stevens 1987, Summers *et al.* 1996), or to a general depression of metabolic rate under these conditions. Based on previous studies with temperate *Dreissena* and *Unionid* species from North America (Aldridge *et al.* 1987, Summers *et al.* 1996) we would have expected the less disturbed L2 animals to show a depression of respiration and the animals from L1 station to be adapted to high sediment loads and thus, less affected. However, the reduction in oxygen uptake in the large L1 individuals under sedimentation is only 20% compared to control respiration and seems to be a preventive response rather than the drastic drop in respiration by 25–70% observed in different *Dreissena* species (Alexander *et al.* 1994, Summers *et al.* 1996). We interpret the response pattern at the impacted L1 station as a behavioural change, presumably due to a learning effect of the animals exposed to frequent sediment runoff plumes. Recently undertaken studies on krill and different ascidians in



Fig. 11. Ventral view of *L. elliptica* after sediment exposure. A large amount of sediment packed in mucus (arrow) is located between the gills (stars).

Potter Cove indicate that those groups are much more sensitive to sedimentation than *L. elliptica* and already respond to concentrations of $< 50 \text{ mg l}^{-1}$ with decreased feeding rates and changes of respiration (V. Fuentes, Torre *et al.* unpublished data). Nevertheless, the bivalves have to transport sediment particles over the gills, cover them with mucus and excrete them through pumping movements (Fig. 11). Higher sedimentation rates that lead to a reduction of aerobic metabolism and increased energy expenses for pseudo faeces production in larger, older animals, which are important for the reproductive output of the population (Abele *et al.* 2009), may therefore in the long run further impact the population structure in addition to the reduced reburrowing ability and injury tolerance.

A small number of studies in the Arctic investigated the abundance and community structure of benthic fauna with respect to the sedimentation rate at different distances from the glacier or fjord heads (Syvitski *et al.* 1989, Holte & Gulliksen 1998, Wlodarska-Kowalczyk *et al.* 2005). A decrease in fauna biomass and richness close to the glaciers in areas with high sedimentation, as well as a diminished abundance of filter feeders and surface detritus feeders is found in these areas. This is consistent with lower numbers/density of *L. elliptica* in the sediment discharge zone in

Potter Cove. Detailed studies of the physiological effect are, however, also missing in the Arctic region.

Taken together, *L. elliptica* seems to be well adapted as a species to survive under changing environmental conditions with respect to high sedimentation rates and ice scouring frequencies. Sediment effects on respiration are marginal and possibly protective as the decrease in respiration and thus presumably filtration rate will reduce the uptake of inorganic particles during times of high environmental sediment loads. Further, at least the small individuals can rapidly reburrow into the sediment and endure severe injury. This might however change when seawater temperatures increase. Peck *et al.* (2004) found *L. elliptica* to suffer 50% failure to reburrow into the sediment at 2–3°C and a complete loss at 5°C. The combined effect of increasing temperature, sediment load and ice scouring frequencies therefore threaten to change the population structure and abundance of *L. elliptica* if climate change proceeds. In the Arctic small surface deposit feeders such as the bivalves *Yoldiella* sp., *Portlandia arctica* (Gray) or *Nuculuna tenuis* (Montagu) were found in high sedimentation areas fronting glaciers (Syvitski *et al.* 1989, Holte & Gulliksen 1998, Wlodarska-Kowalczyk *et al.* 2005). Possibly in Antarctic environments like those of Potter Cove, detritus feeders such as polychaetes or the bivalve *Y. eightsi* (Davenport 1988) will take over in places where *L. elliptica* will be reduced in abundance. This will change the shallow Antarctic ecosystems as the larger and deeper burrowing *L. elliptica* has a much greater effect for pelago-benthic carbon flux through biodeposition of material into deeper sediment horizons (Ahn 1993, Momo *et al.* 2002).

Acknowledgements

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Supplemental data

Underwater photographs of sites – see www.journals.cambridge.org/jid_ANS.

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5. ADDITIONAL RESULTS

This section presents additional results from the investigation of the *Laternula elliptica* molecular expression response under food restriction, an initial characterization of selected genes putatively involved in the animals functions of growth and/or metabolism as well as an investigation of tissue proliferation rates.

5.1 Candidate genes

Using key word search, a number of contigs with sequence similarity to known genes with a potential role in the maintenance of cellular homeostasis, proliferation and metabolic processes of *L. elliptica* was identified (Tab. 1 and 2). These include functional components of the cell cycle/cellular renewal (cyclin B and cyclin D), growth and utilization of polysaccharide or polypeptide substrates (α amylase, cathepsin B), intracellular [ATP] regulation (AMP-activated protein kinase, AMPK), development and maintenance of cellular homeostasis (ferritin, see manuscript III) or induction of autophagy (beclin 1). As these candidate genes have previously been reported to play a role in physiological response to environmental conditions in other organisms, they were selected from the database in order to further characterize the *L. elliptica* gene expression under food restriction as well as tissue-specific expression pattern by quantitative *real time* PCR. The RNA sequence database project was deposited as sequence read archive (SRA) study ERP001323 and project number PRJEB33 at the European Nucleotide Archive (ENA). Assembled RNA sequences were deposited in the ENA as Transcriptome Shotgun Assembly (TSA) and accession numbers are given throughout the text or tables.

Table 1: Selected contigs putatively involved in *Laternula elliptica* metabolism. The information regarding the best BLAST match within the UniprotKB/Swissprot and NCBI/nt-blast databases is given for each contig. Further contig details including sequence length and domain structure are listed in table 2. Genes/contigs were investigated for differential expression in stress treated *L. elliptica* individuals by qRT-PCR (for ferritin see Manuscript III).

Category/ Gene name	ENA Accession	UniprotKB/Swissprot				NCBI, nt			
		Accession	Description	Organism	E-value	Accession	Description	Organism	E-value
α Amylase	HE820924	P91778	α Amylase	<i>Pecten maximus</i>	0	Y08370.1	α Amylase	<i>Crassostrea gigas</i>	0
Cyclin B	HE820925	P24862	G2/mitotic-specific cyclin-B	<i>Patella vulgata</i>	1E-112	X58358.1	Cyclin B	<i>Patella vulgata</i>	2E-134
Cyclin D	HE820926	P49706	G1/S-specific cyclin-D2	<i>Gallus gallus</i>	6E-35	NM_001076372.1	Cyclin D2	<i>Bos taurus</i>	8E-43
Protein kinase AMPK	HE820927	Q8BRK8	5'-AMP-activated protein kinase catalytic subunit alpha-2	<i>Mus musculus</i>	1E-156	DQ825653.1	Protein kinase AMPK alpha subunit 1	<i>Artemia franciscana</i>	0
Cathepsin B	HE820928	A1E295	Cathepsin B	<i>Sus scrofa</i>	1E-111	HM749974.1	Cathepsin B	<i>Crassostrea ariakensis</i>	3E-157
Ferritin	HE804761	P42577	Soma ferritin	<i>Lymnaea stagnalis</i>	3E-70	GQ139542.1	Ferritin-like protein	<i>Pinctada maxima</i>	7E-87
Beclin 1	HE820929	Q4A1L3	Beclin-1	<i>Xenopus tropicalis</i>	3E-37	XM_002414804.1	Beclin, putative	<i>Ixodes scapularis</i>	7E-47

Table 2: Sequence (contig length and number of reads in the transcriptome) and protein domain structure information of selected contigs putatively involved in *Laternula elliptica* metabolic functions. The protein domain structure in the different contigs was deduced from the NCBI conserved domain architecture retrieval tool cDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) and compared to the conserved domain structure for the respective gene as defined by HomoloGene (<http://www.ncbi.nlm.nih.gov/homologene>). Genes/contigs were investigated for differential expression in stress treated *L. elliptica* individuals by qRT-PCR.

Gene	ENA Accession	Contig length (bp)	Nr. of reads	Domain-containing longest ORF	Domains expected	Domains identified
α Amylase	HE820924	522	71	522	AmyAc_family, α -Amylase_C	AmyAc_family, Alph (α -Amylase_C)
Cyclin B	HE820925	1008	4	233	CYCLIN (2x)	CYCLIN (2x)
Cyclin D	HE820926	521	3	111	CYCLIN (2x[Coelomata]/1x[<i>Drosophila melanogaster</i>])	CYCLIN (1x)
Protein kinase AMPK	HE820927	1104	4	356	S-TKc or PKc-like (alpha catalytic subunit)/ AMPKBI, E_set (beta-1 non-catalytic subunit)	PKc-like
Cathepsin B	HE820928	1827	465	358	Propeptide_C1, Peptidase_C1	Propeptide_C1, Peptidase_C1
Ferritin	HE804761	1876	2416	175	Ferritin-like	Euk_Ferritin
Beclin 1	HE820929	452	2	72	MT, APG6	None (Pfam: APG6)

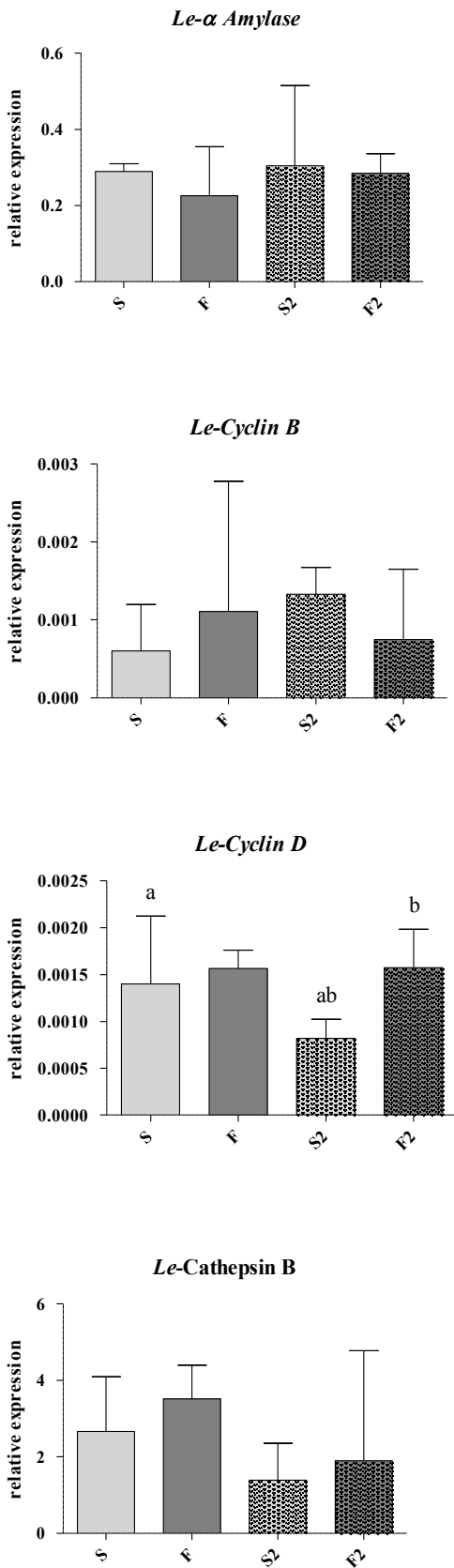
5.2 Effect of starvation on digestive gland gene expression

Expression levels of candidate genes putatively involved in the maintenance of cellular homeostasis, proliferation and metabolic processes were investigated in *L. elliptica* sampled from the experimental food restriction treatment (3.2.3) in a size- (two non-overlapping size classes of larger and smaller individuals) and time-dependent manner (i.e. on day 23 and 44 after starting the nutrition treatments). Digestive glands were analyzed, because they contain major components of the digestive system and metabolism of the animals. In most cases, the two-way ANOVA revealed no significant effects of the different feeding conditions on digestive gland gene expression (Fig. 1, Tab. 3). The only difference between both treatments “food” and “starvation” was detected in digestive glands of the young individuals. After 44 days under starvation, expression levels of *Le*-cyclin D, a cell cycle protein, which is highly conserved between organisms, was significantly lower (1.7-fold) compared with expression levels after 23 days of starvation. In contrast, young animals which were fed for 44 days maintained constant gene expression and *Le*-cyclin D transcripts in these animals were significantly higher (1.9-fold) compared to animals from the 44 day starvation treatment. Therefore, a reduction of the amount of *Le*-cyclin D transcripts is indicated under 44 days starvation treatment. As this downregulation of *Le*-cyclin D in small specimens under food restriction is consistent with a similar (although non-significant) trend in large specimens of the same treatment, expression of this cellular proliferation marker in digestive gland tissues is indicated to be linked to the availability of food.

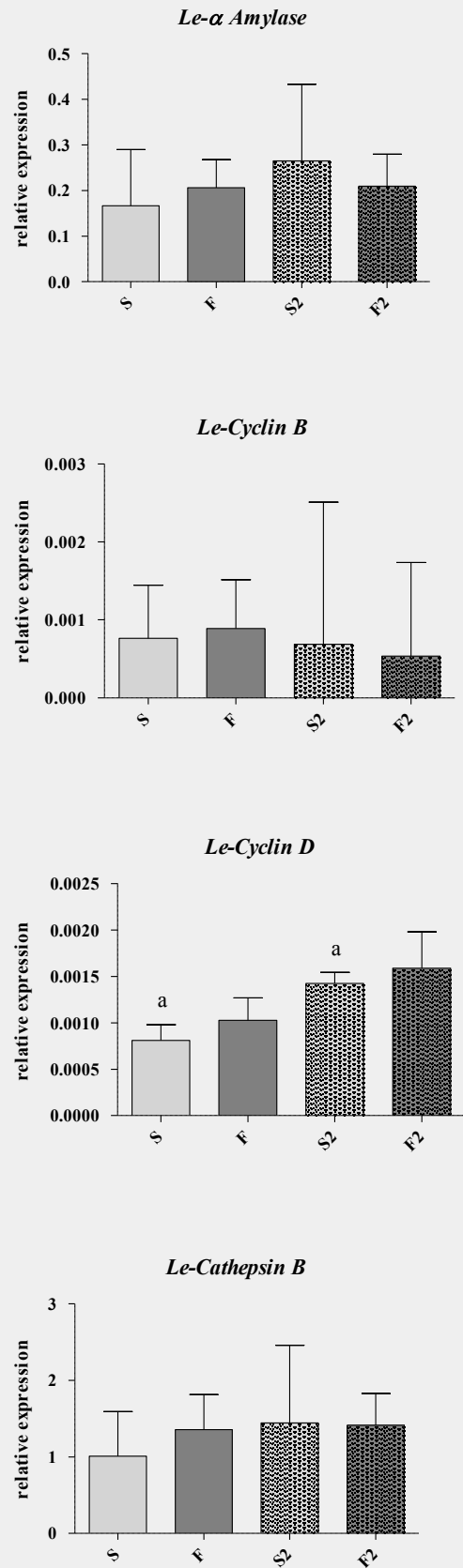
Transcriptional levels of *Le*-beclin 1, a protein with putative key functions in coordination of autophagy, an adaptive mechanism of degradation of cytoplasmic organelles and proteins, nearly doubled in young starved individuals between the first and the second sampling date. This effect failed to reach significance in a two-way ANOVA, but, however, was not observed in the group of young fed animals, pointing to a possible induction of autophagy under periods of environmental food restriction.

On the one hand, the lack of strong responses under food restriction indicates treatment-times non-sufficient to induce severe starvation effects, on the other hand this can be ascribed to the adaptation of *L. elliptica* to the Antarctic environment including specifically increased capability to survive under unfavourable environmental conditions (see **discussion**).

SMALL



LARGE



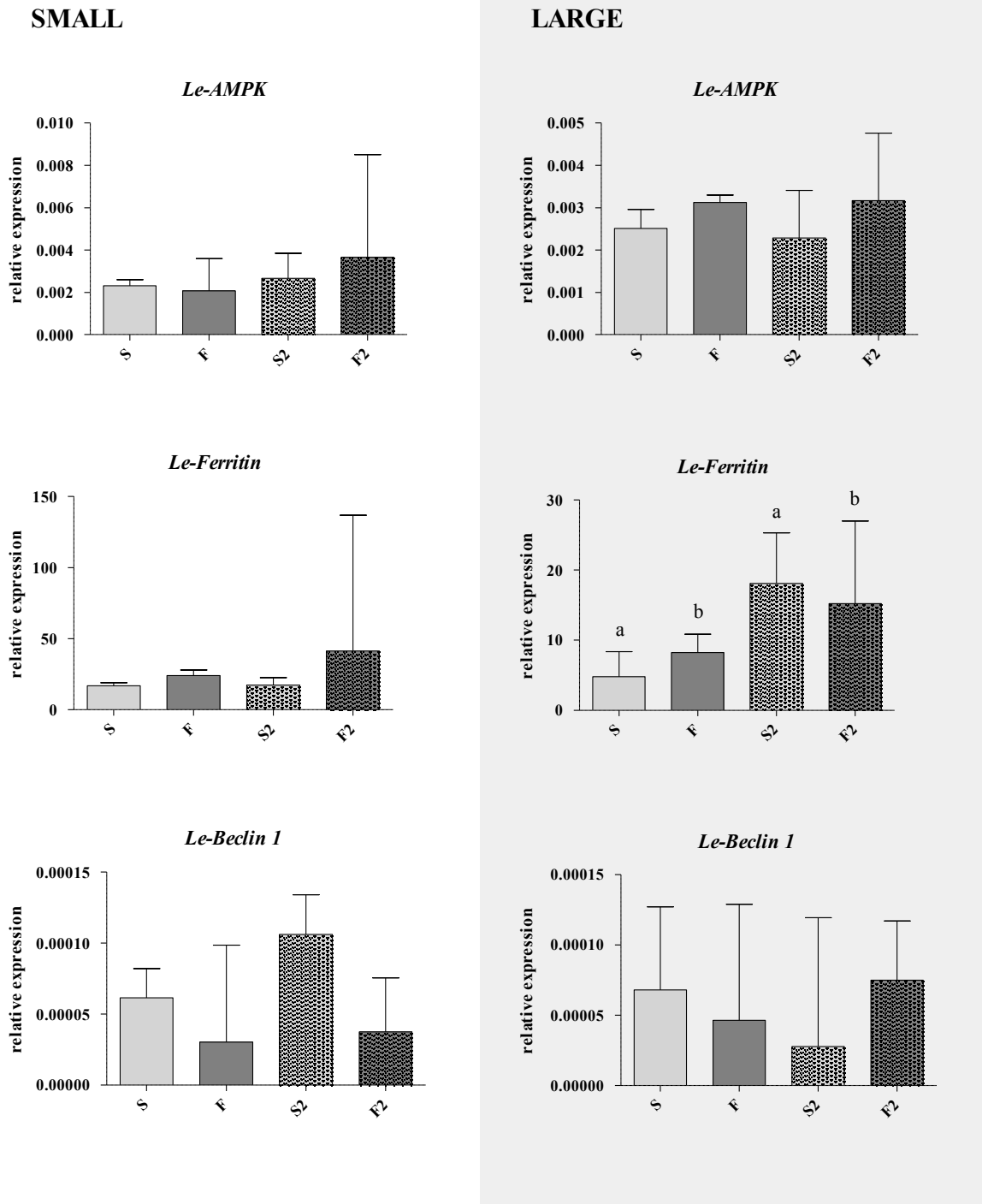


Figure 1: *Laternula elliptica* digestive glands: Expression levels of selected transcripts putatively involved in metabolic responses measured by qRT-PCR in different sized/aged *L. elliptica*. Individuals were sampled after 23 days of “food” (F) and “starvation” (S) treatment, additional samples were taken after 44 days of “food” (F2) and “starvation” (S2). Expression levels were normalized using actin as the reference gene. Data are presented as the median and interquartile range, N=6-8 individuals per group. Groups with similar letters are significantly different from each other with $p < 0.05$ (two-way ANOVA with Bonferroni post hoc test).

Table 3: Effects of different feeding conditions on gene-expression in digestive glands of different sized/aged *Laternula elliptica*. After laboratory acclimation, individuals were kept under 23 days of “food” and “starvation” conditions, as well as 44 days of “food” and “starvation”. Data were obtained by *real time* PCR and calculated using actin RNA as a reference. Significant effects between groups (N=6-8) and interactions were revealed by two-way-ANOVA and are highlighted by *** (p<0.001), ** (p<0.01) and * (p<0.5). Data highlighted by # were analysed by t-test with Welch correction. N.s. = not significant.

Gene	Young Individuals			Old Individuals		
	Treatment	Time	Interaction	Treatment	Time	Interaction
<i>Le</i> -AMPK	n.s.#	n.s.#	n.s.#	n.s.	n.s.	n.s.
<i>Le</i> - Amylase	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Le</i> - Beclin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Le</i> - Cathepsin B	n.s.	*	n.s.	n.s.	n.s.	n.s.
<i>Le</i> -Cyclin B	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Le</i> -Cyclin D	*	n.s.	*	n.s.	***	n.s.
<i>Le</i> -Ferritin	n.s.#	n.s.#	n.s.#	n.s.	***	n.s.

5.3 Tissue expression profiling of *Laternula elliptica* ferritin

Ferritin is one of the principal proteins for biological storage of intracellular iron and metabolism. As filter feeders can accumulate considerable amounts of metals from the environment, characteristics of putative ferritin expression in *L. elliptica* deserve special attention.

Strikingly highest *L. elliptica* digestive gland relative expression levels of all genes investigated in this study were found for transcripts of *Le*-ferritin (Fig. 1). In order to address a possible functioning of *Le*-ferritin *in vivo*, the specific expression was determined within the *L. elliptica* tissue panel, consisting of hemocytes, mantle, siphon, foot, gills and digestive glands of untreated animals. As levels of 18s ribosomal mRNA appeared much more uniform across tissues compared with actin, they were used as housekeepers. *Le*-ferritin expression was detected in all tissues (Fig. 2). The highest relative expression was found in digestive glands, followed by hemocytes, gills and foot. The lowest levels were found in siphon and mantle, which had 71-fold and 5-fold lower values (median) compared to digestive glands respectively (Fig. 2). The multifold higher expression of *Le*-ferritin in gills, digestive glands and hemocytes might reflect the physiological function of these components in bivalves and go hand in hand with the proposed way of *L. elliptica* uptake and transport from ingested food

and water via structures of filter feeding (gills) and digestion (digestive glands) (manuscript II) and internal transport via the hemolymph/hemocytes system.

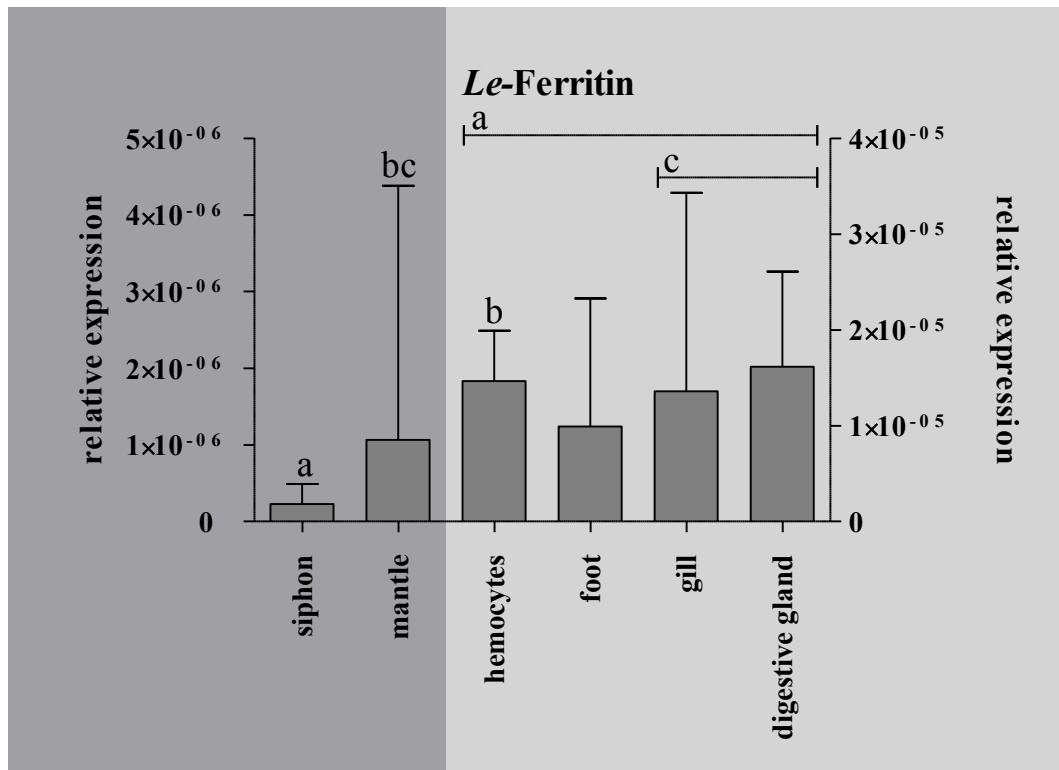


Figure 2: *Laternula elliptica* mRNA expression levels of *Le-ferritin* in different tissues and hemocytes analysed by qRT-PCR. Values were normalized using 18s ribosomal RNA as the reference gene. Data are presented as median and interquartile ranges of relative expression values, N=5 per group (hemocytes: 6, siphon: 4). Columns, or groups of columns, with similar letters are significantly different from each other ($p < 0.05$).

5.4 Tissue expression profiling of *Laternula elliptica* cyclin B and cyclin D mRNA

Cyclin B and cyclin D are addressed as regulatory subunits in controlling the mitotic cell cycle. As a first step towards further characterizing of growth in *L. elliptica* both transcripts were used as markers for proliferative phases and specific expression was analyzed in different tissues. Cyclin D is involved in G₁/S-phase-transition and starts to accumulate at mid-G₁-phase after extracellular growth signaling (Blagosklonny and Pardee 2002). The relative mRNA expression of *Le-cyclin B* and *Le-cyclin D* was determined in hemocytes, mantle, siphon, foot, gill and digestive gland of untreated, old animals. Expression of both genes was detected in all tissues (Fig. 3). The highest expression of *Le-cyclin B* was found in siphon,

lower values in hemocytes, mantle, foot and digestive gland and lowest expression in gills. Gill expression was 5-fold lower than in siphon tissue. *Le*-cyclin B showed multifold higher levels of expression, compared with the values detected for putative cyclin D. Also the transcripts designated as cyclin D showed tissue-specific expression values. Here highest relative expression was found in hemocytes and lowest values in digestive glands, which were 16-fold lower (median) compared with the level in hemocytes, indicating high proliferation activity in hemocytes.

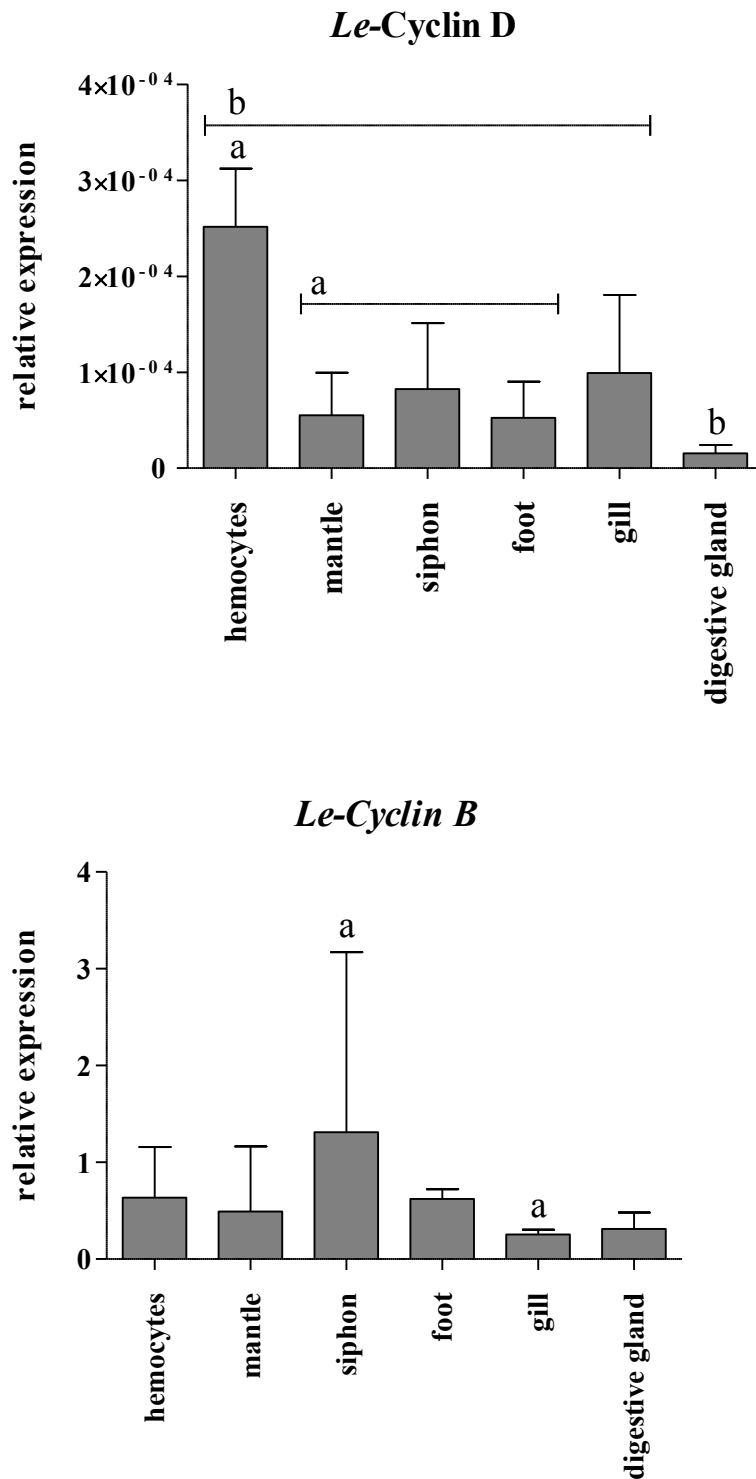


Figure 3: *Laternula elliptica* mRNA expression levels of *Le-cyclin B* and *Le-cyclin D* in different tissues and hemocytes analysed by qRT-PCR. Values were normalized using 18s ribosomal RNA as the reference gene. Data are presented as median and interquartile ranges of relative expression values, N=5 per group (hemocytes: 6, siphon: 4). Columns, or groups of columns, with similar letters are significantly different from each other ($p < 0.05$).

5.5 Seasonal expression pattern of selected candidate genes

As a first step towards further characterizing growth in *L. elliptica* the seasonal expression of selected genes (*Le*-cyclin B, *Le*-cyclin D, *Le*- α amylase, *Le*-cathepsin B) putatively involved in metabolism or growth was analyzed in digestive glands of old individuals collected after the Austral winter and during late Austral summer (Fig. 4).

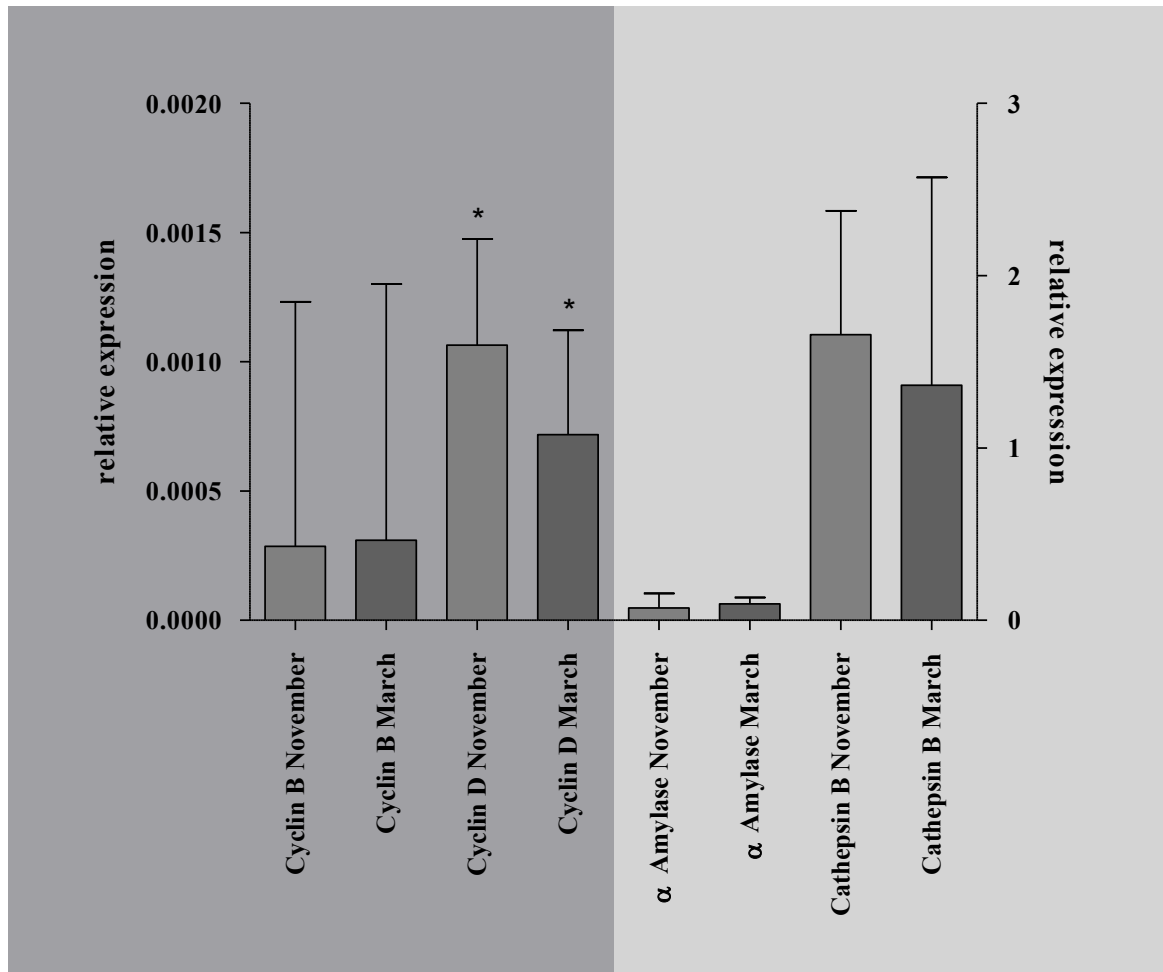


Figure 4: Gene-expression levels in digestive glands of *Laternula elliptica* collected after the Austral winter (*winter*, 6th November 2008) and during late Austral summer (*summer*, 5th March 2009). Animals were classified as “old” with (mean and SD) 80 ± 5 mm shell length. Values in median and interquartile range (N=7 animals per group). Significant differences after Mann Whitney *U*-test between groups * with ($p < 0.05$).

Only for *Le*-cyclin D seasonal differences in expression with higher values in November were found. Transcript levels were significantly (1.5-fold) higher accumulated in digestive glands collected after winter compared with those collected during summer, possibly pointing to high

growth activity during the chosen sampling time at the beginning of the summer season (November).

5.6 Tissue proliferation rates (histological)

Within 5 days of BrdU incubation, incorporation of BrdU was detected in all investigated tissue types (Fig. 5). Young *L. elliptica* displayed significantly higher proliferative activity (in mantle up to 68-fold higher mean values), siphon (9-fold higher mean value) and muscle tissue (up to 265-fold higher mean value) than old individuals (Fig. 6), pointing to higher somatic growth in younger individuals. No significant difference between size/age groups was found in foot and gill tissue (Fig. 6). The overall highest proliferation value was detected in mantle tissue of young animals collected in summer close by station L1, with 8.6 ± 3.8 % of all cells dividing within 5 days compared to 0.13 ± 0.25 % in the corresponding old animal group. Highest tissue-specific proliferation rates in old *L. elliptica* were found in the gills, pointing to an actively proliferating tissue. Contrary, mantle, siphon, muscle and foot display very little if any cell division in a 5 day recording interval even in summer. Seasonal differences were only detected in mantle tissue of younger animals, with a mean proliferation rate of 8.63 ± 3.83 % (March: 1.7 % per day) of cells dividing within 5 days in summer compared to 2.21 ± 1.91 % in winter (November: 0.4% per day). Due to the morphological characteristics and consistency of digestive gland and ganglia/nervous tissue we were not able to obtain an appropriate section quality of these tissues by cryo-sectioning technique and could not determine proliferation rates in these tissues.

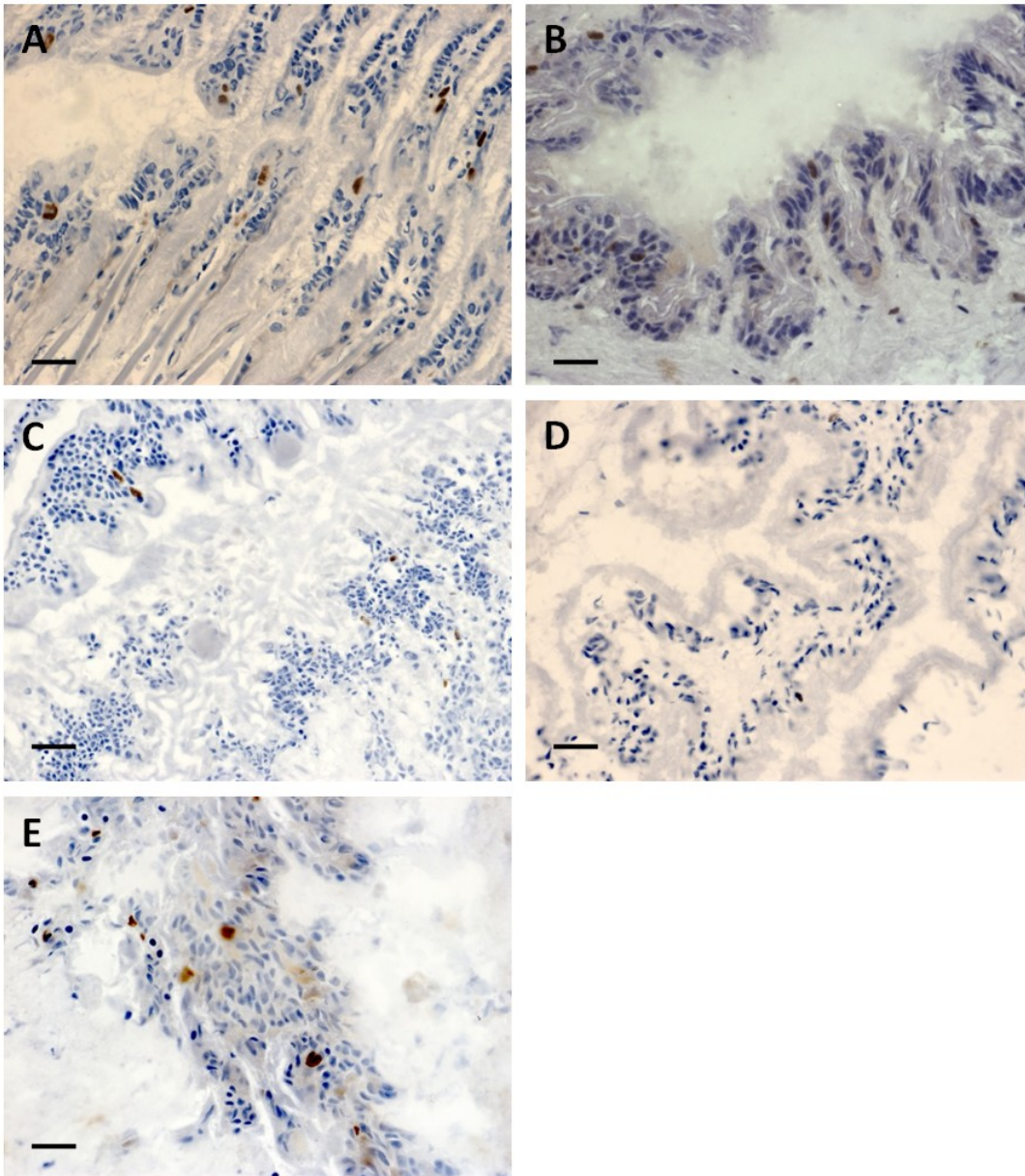


Figure 5: Cryosections of *Laternula elliptica* gill (A), mantle (B), siphon (C), foot (D) and adductor muscle (E) after 5 days of BrdU incubation. To visualize cell nuclei without incorporated BrdU (blue appearance), sections were lightly stained with hematoxylin. Nuclei executing replicative DNA synthesis appear dark brown after the immunohistochemical detection of incorporated BrdU. Scale bar = 20 μm .

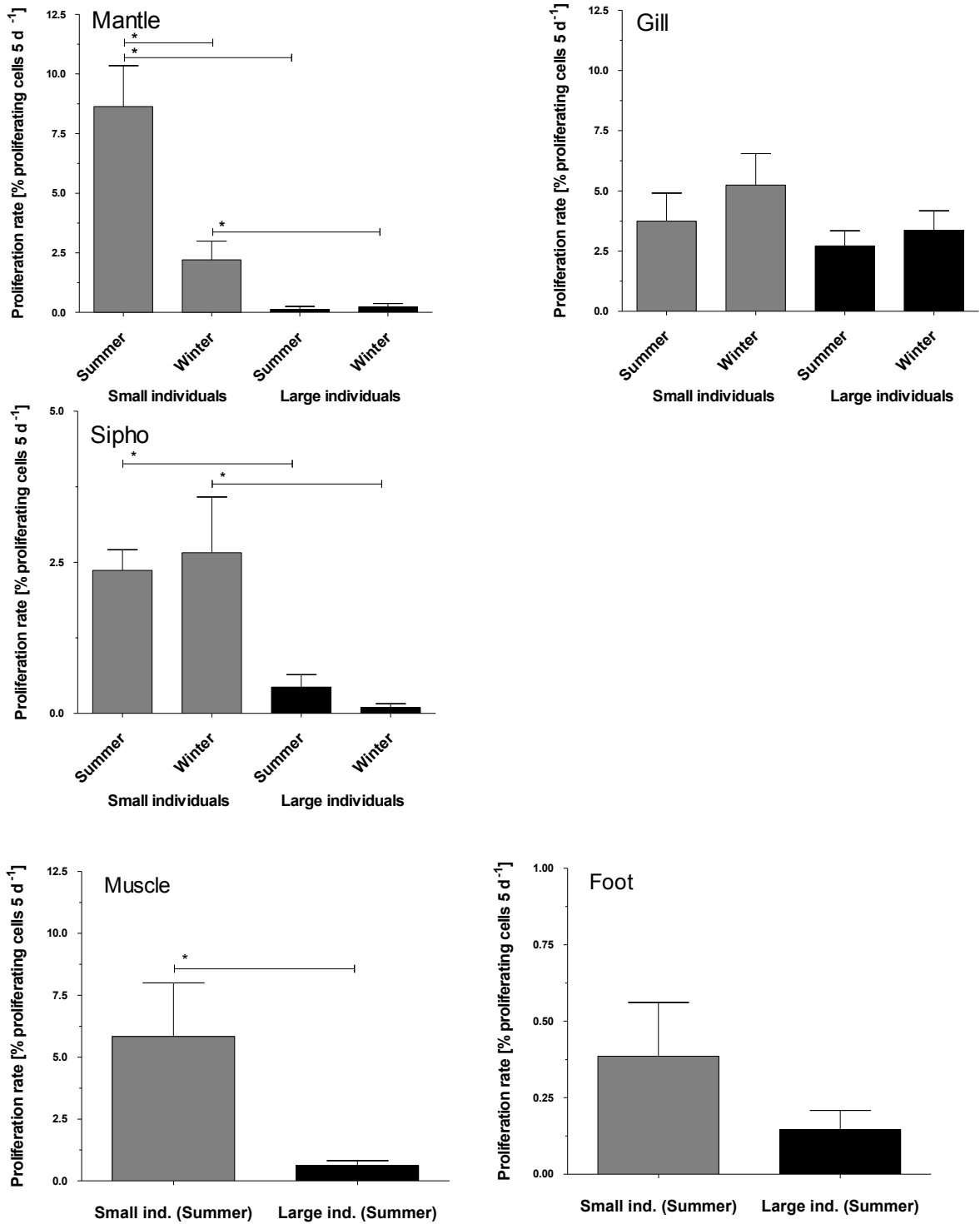


Figure 6: Proliferation rates [% proliferating cells 5d⁻¹] in different tissues of different sized animals (80 ± 5 mm, 46 ± 5 mm) of *Laternula elliptica*, collected after the Austral winter (winter, November) and during late Austral summer (summer, March). Values in means ± S.E.M. (N=5-6 animals per group) after 5 days of BrdU-incubation. Significant differences between groups * with (p<0.05).

6. DISCUSSION

The present work addresses physiological responses of Antarctic bivalves *Laternula elliptica* to current trends of complex environmental changes at the Western Antarctic Peninsula (WAP) with superimposition of warming, increase of ice scouring and inorganic turbidity. In this chapter, the most important results will be discussed by outlining specific physiological reactions on different levels of the *L. elliptica* organismic organization.

6.1 Physiological responses of *Laternula elliptica* to high turbidity and ice scouring

Within the first part of this study bivalve behavior and general metabolism, as well as cellular markers were age-specifically investigated in two populations sampled from the field. As laid out in manuscript **IV**, *L. elliptica* populations investigated in the present study experience different levels of summer water column turbidity (suspended particulate matter, SPM) and differing frequencies of ice scouring impacts. These parameters are depending on geocharacteristics of the different locations of *L. elliptica* including distance from major glacial runoff streams, hydrographic setting (currents) and depth distribution. Immediate effects of daily air temperature changes on SPM values which were observed at the *L. elliptica* sampling location close to a major runoff are in line with Dominguez and Eraso (2007) which previously linked peaks of air temperature warming to increasing glacial sediment discharge in the region of King George Island. Recent findings of Bers et al. (2012) pointed out climatic characteristics such as the Southern Annual Mode (SAM) and El Niño events as explanatory variables directly influencing glacier melting and total suspended particulate matter (TSPM) in Potter Cove. In the present study, effects of glacier discharge appear to immediately reach the benthic community, as the most distinct and significant differences were found by comparing near-bottom SPM values at both investigated locations of *L. elliptica* peaking directly following short-term air temperature warming. Overall, data presented in manuscript **IV** document that both Potter Cove field sampling stations chosen for this study can be regarded as representative of two distinctly different environmental stress scenarios. These are expected during present climate alterations acting on glacial disintegration and turbid runoff and finally on the local benthic populations.

6.1.1 Short-term effects of high inorganic turbidity

Deduced from the results of this study, *L. elliptica* populations have a considerable capacity to tolerate periodically increasing turbidity and can survive immediate consequences of ice scouring, however, age is an important factor in the species stress tolerance.

A reduction of respiration and filtration under high sediment runoff was experimentally indicated for older age groups originated from the more exposed sampling site (manuscript **IV**). It is not clear, how this might rank in overall biological impact when assigned to the environment. Although various marine bivalves have been shown to reduce respiration under periods of high inorganic turbidity (Aldridge et al. 1987a; Grant and Thorpe 1991; Alexander Jr et al. 1994), the basic physiological mechanisms causing this drop are unclear. In general metabolic depression to overcome periods of unfavourable external conditions is known in bivalves (Strahl et al. 2011; Abele et al. 2009; Sokolova et al. 2012).

Also *L. elliptica* apparently is able for a general metabolic reduction in order to minimize energetic costs during Antarctic winters. The latter manifests by periods of siphon closure, reduced pumping activity, heart arrhythmia and depression of oxygen consumption (up to 3/3.7-fold) (Brockington 2001; Brey et al. 2011; Morley et al. 2007). However, the respiration reduction of *L. elliptica* experimentally induced by inorganic turbidity in the present study comprised only ~20% compared to control respiration and no increase of siphon closure (interrupted respiration patterns, IPs) was detected (manuscript **IV**). Moreover, respiration rates of similar aged animals from the less exposed sampling site as well as younger age groups were not affected. Therefore decreasing respiration in animals from the highly exposed habitat could be interpreted as adaptive reduction of ventilation during peaks of high turbidity. Thus an excessive uptake of inorganic particles can be partially avoided which might help *L. elliptica* to reduce the expenditure for particle sorting, pseudofaeces production and removal (Fig. 16) (Alexander Jr et al. 1994; Madon et al. 1998; Shin et al. 2002).

Hence, rather adaptive responses instead of sequentially declining activity levels under persisting runoff conditions might indicate a broad capacity to tolerate turbidity as additionally seen on the animals specific local burrowing behavior (manuscript **IV**). This physiological parameter was used as activity marker in several bivalves and has been viewed as both measure of adaptive ability under environmental impact as well as indicator of total health (Byrne and O'Halloran 2000; Joyner-Matos et al. 2009; Pearson et al. 1981). In spite of higher runoff-disturbance levels close to the glacial runoff area investigated for this study, individual burrowing activities were actually higher in *L. elliptica* from the exposed

environment possibly pointing to a behavioural adaptation due to more frequent needs to adjust within the sediments.

6.1.2 Long-term effects of high inorganic turbidity

Nevertheless, a long-term exposure (years) to runoff conditions apparently affects the animals physiological state. In particular when comparing older bivalves from both stress scenarios, the detected differences in physiological performance (respiration) and cellular markers (lipofuscin, protein carbonyls, intracellular accumulated metals) indicated local differences in extrinsic impact that might sequentially act on the physiological state of the animals. As an example, lifelong environmental sediment exposure can go hand in hand with a progressive gill damage and impairment of ciliary activity (Stevens 1987; Shin et al. 2002). This may be one factor causing impaired respiration found in *L. elliptica* from lifelong exposure at the habitat close to the runoff when experimentally again exposed to sediments (manuscript IV). According to Sokolova et al. (2012), however, situations of moderate physiological stress in molluscs are characterized by impaired aerobic metabolism and increasing maintenance costs. Consequently energy homeostasis has to be achieved at the expense of physiological processes such as growth, reproduction or energy storage. Accordingly, sediment exposure was shown to cause a deficit on energy budget in different bivalve species which in bivalves finally leads to inhibited physiological parameters such as growth or increased mortality (Wilson et al. 1996; Grant and Thorpe 1991). *Mya arenaria*, a temperate species morphologically very similar to *L. elliptica* reacted by reduction of respiration, stopped increasing tissue weight and utilized energy reserves when experimentally treated by sediments (Grant and Thorpe 1991). Hence, long-term inorganic turbidity exposure of *L. elliptica* accompanied by a periodical reduction of the animals' metabolic rates and possibly affected filtration (manuscript IV) can in the long run lead to both physiological stress (manuscript II) and a decrease of physiological fitness. In keeping with this, accumulation of the cellular marker lipofuscin was higher in nervous tissues of older *L. elliptica* sampled in the present study at the more exposed station (L1). Lipofuscin is regarded as an undegradable and not removable final product of stress induced oxidative damage and hence was considered a biomarker for long-term concerted impact of different stressors occurring at this station (manuscript II). Increasing intracellular lipofuscin deposition can contribute to disturbance of cellular and physiological functioning and senescence, for example by direct inhibition of proteasomal functioning and autophagocytotic capacity (for review see Brunk and Terman 2002; Terman and Brunk 2004; Sitte et al. 2000).

Possible consequences of this concerted impact include a depression of the overall lifetime growth at the more disturbed station L1. A long-term adaptive strategy of Maxwell Bay (King George Island) *L. elliptica* to cope with increased glacial runoff of the last decades apparently is an acceleration of somatic growth in younger individuals (≤ 10 years) at the expense of growth and maximum size in later life (Brey et al. 2011). Reduced overall growth in older individuals might explain results of the lipofuscin detection in the present study. Lipofuscin additionally accumulates with chronological age in *L. elliptica* and also other bivalves (for review see Philipp and Abele 2010). Higher lipofuscin concentrations found in the larger/older size class from L1 (disturbed) compared to similar sized individuals from L2 (less disturbed) indicate that in spite of similar size the individuals from L1 might have been older. This might indicate a depression of growth rates under higher disturbance at station L1. However, this remains speculative, as growth rates could not be determined as the *L. elliptica* shells from the field station comparison were lost in a fire on the Argentine ice breaker ARA Almirante Irizar during transport. Indeed, preliminary data pointed out individuals of the more disturbed sampling location L1 may grow less consistently compared to individuals from the less disturbed sampling station L2 (investigated age class of 10-19 years) (K. Jenkins, unpublished data).

Knowledge of seasonal growth characteristics in *L. elliptica* can contribute to an understanding of long-term consequences induced by turbid runoff and ice scouring which is predominantly occurring during summer. Cellular proliferation rates investigated in individuals collected during March and November indicate a major growth period of the species during Antarctic summer. Seasonal differences, however, were only significant in mantle tissues (epithelia) of younger individuals (**Additional Results**). Findings of distinctly differing seasonal proliferation rates agree with distinctive annual growth pattern formation inside the *L. elliptica* shell umbo (see also Brey and Mackensen 1997; Brey et al. 2011; Ahn and Shim 1998). In bivalves the mantle is responsible for shell formation and especially epithelial zones apparently have key functions for the whole mantle's differentiation and growth (Fang et al. 2008). Studies about the seasonal dynamics of cell proliferation in epithelia of somatic tissues of various bivalves revealed a similar seasonal pattern (Leibson and Frolova 1994; Zaldibar et al. 2008). These studies pointed out two different seasonal states of epithelial proliferation in bivalves, of which the first is characterized by a resting period in winter and early spring, followed by a burst of cell division in summer. Although results in *L. elliptica* might have been expected, characteristics of growth control in this species are not clear as seasonal temperatures vary only marginally and are much more

uniform compared to temperate waters. Distinctive seasonal growth rates can in part be explained by a close coupling to the seasonal planktonic primary production anticipated for various species of the Antarctic benthos including *L. elliptica* (Brockington and Clarke 2001; Ahn et al. 2001; Schloss et al. 2012). As the seasonal variability of water temperatures is only marginal, partially only punctual availability of food during summer seems to be a major key factor governing growth in *L. elliptica*. Indeed, *L. elliptica* apparently has a high capacity for a rapid response of growth to food and tissue mass values were shown to be positively correlated with chlorophyll concentrations rather than water temperature (Brockington 2001; Ahn et al. 2003). On a molecular level, this might go hand in hand with findings that in spite of the short experimental food restriction treatment not capable of affecting severe starvation effects (manuscript **III**, **Additional Results**) most prominent transcription regulation was found in genes putatively involved in cellular development (**Additional Results**). A close coupling of growth rates to summer food supply appears critical considering possible consequences of summer turbid runoff on feeding conditions. Indeed, deduced from reconstruction of *L. elliptica* decadal growth variability Brey et al. (2011) ascribed an overall decreased lifetime production (such as shell growth, somatic production, gonad production) to increasing maintenance costs caused by increasing turbid runoff at Maxwell Bay (KGI).

On the one hand, filter feeding rates might be affected as also indicated by *L. elliptica* respiration reduction under sediment impact. However, the relationship of food uptake or assimilation under increasing turbidity is not quantified in *L. elliptica* and needs further investigation. On the other hand, it is not clear, to what extent massively increasing glacier runoffs during summer affect the local availability of planktonic and benthic microalgae, the *L. elliptica* populations rely on. Moreover, the relative importance of different food sources, including resuspended detritic material, that might be alternatively utilized by the Antarctic benthos (Ahn 1997; Tatián et al. 2008; Gili et al. 2001), has to be addressed in the future, to estimate runoff-induced consequences for *L. elliptica* food sources and food supply of animals in disturbed areas. However, increasing turbid runoff can reduce irradiance attenuation and salinity, increase stratification within the water column and therefore alter conditions for algal growth (Dierssen et al. 2002; Schloss and Ferreyra 2002; Montes-Hugo et al. 2009; Schloss et al. 2002).

Indeed, *L. elliptica* from areas less impacted by mineral suspension might ingest proportionally more digestible food particles as indicated by overall higher metal concentrations that were found in tissues of older animals from station L2. Contrary to the initial hypothesis of this work, *L. elliptica* does not seem to record the higher runoff of turbid

melt water by an increased intracellular accumulation of metals (manuscript II). Instead, the results from metal measurements in sediments and tissues indicated metal uptake in *L. elliptica* is rather depending on small scale local characteristics of the geochemical sedimentary environment, age of the animals and bioaccumulation from ingested food or water. In particular higher enrichment of metals such as Cd found in *L. elliptica* filter feeding components including gills and digestive glands from older L2 individuals compared to older L1 individuals can be attributed to higher rates of bioaccumulation due to higher filtering activity and/or food supply. Major food sources of the animals (Antarctic diatoms or detritus of benthic macroalgae) are known to accumulate metals and in particular high amounts of Cd from the upwelling seawater at the WAP (deMoreno et al. 1997; Bargagli et al. 1996; Bargagli 2001). Lower TOC/TN ratios found in the top sediment layer at the less exposed sampling site L2 pointed towards higher available amounts of freshly deposited organic matter and probably better feeding conditions at this site (manuscript II) which might lead to the higher Cd tissue levels.

Higher levels of protein carbonyls found in *L. elliptica* tissues from the less exposed station L2 pointed to higher rates of oxidative protein modification due to oxidative stress. Findings were partly attributed to higher oxidative damage possibly caused by the higher tissue contents of metals such as Cu, Fe or Cd that can contribute to catalysis of oxidative damage (Prousek 2007; Valko et al. 2005). Reasons for observed differences between L1 and L2 animals may, however, be caused by many stressors as protein carbonyls are rather general markers of oxidative stress (Ansaldo et al. 2007; Park et al. 2008). The opposed results observed for both cellular oxidative stress markers (lipofuscin and protein carbonyls) most certainly result from the fact that lipofuscin is regarded a non-degradable final product of oxidative damage that accumulates preferentially in postmitotic tissues such as nerves (Brunk and Terman/Terman and Brunk). In contrast, oxidized proteins arising from oxidative stress can be rapidly metabolized in proliferating tissues such as the mantle (Strahl and Abele 2010). Furthermore, metabolism of *L. elliptica* appears to be mainly based on protein turnover (Brockington 2001; Ahn et al. 2003; Rodrigues et al. 2007). Thus, concentrations of oxidative damaged proteins in mantle tissues rather reflect a momentary steady state level of both oxidative stress and protein turnover than a marker of longer-term stress.

To summarize, results received from comparison of respiration rates and cellular markers in animals of both field sampling stations suggest that inorganic turbidity primarily affects *L. elliptica* after several years of exposure. There is evidence for metabolism (respiration) to be affected, as well as for lifelong higher levels of oxidative stress. The animals do not record

higher runoff by increased intracellular accumulation of metals, however, lifelong increasing accumulation from the environment is indicated. Deduced from these data, *L. elliptica* may be well adapted as a species to survive under moderate increasing runoff conditions even though accelerating runoff might contribute to decreasing physiological performance particularly in older specimens.

6.2 Species specific responses under high turbidity and ice scouring

6.2.1 Sedimentation

Immediate/acute effects of high sediment loads on the *L. elliptica* metabolism were marginal. It appeared that there is considerable variation in respiration response and presumably also tolerance level to turbidity when comparing *L. elliptica* with other groups of filter feeders (ascidians) from the same environment (Potter Cove). These in part already start increasing respiration under sediment concentrations of 5-50 mg L⁻¹ which was attributed to extra costs arising by processing of enlarged fractions of inorganic particles and started to diminish dramatically in abundance during the last 15 years (Torre et al. 2012). *L. elliptica* appears to be more tolerant to concentrations ≥ 50 mg L⁻¹ and able to adapt metabolism or behaviour under long-term turbidity and additional ice scouring. This may be one reason why in spite of increasing sediment runoff *L. elliptica* is a dominating nearshore biomass component in Potter Cove that occurs in shallow habitats adjacent to major glacial runoff zones (manuscript IV, Sahade et al. 1998).

Individual density in *L. elliptica* populations investigated in the present study was, however lower close to the runoff compared to the less disturbed population. Long-term effects of runoff can sequentially deteriorate the health of *L. elliptica*, therefore contributing to populations abundance. On the population level, responses to sublethal environmental impacts are complex and are supposed to be determined by long-term changes in multiple ecological factors rather than by separated immediate effects (Peck et al. 2004b). Nevertheless, changing cellular and/or metabolic performances in molluscs exposed to a stressor can be used to approximate the physiological limits of tolerance (Sokolova et al. 2012). Hence, the combination of acute modifications under stress (e.g. reduced respiration rates) and accelerated cellular oxidative damage accumulation (lipofuscin) in long-term exposed individuals together indicate that animals reach a physiological limit of turbidity tolerance.

Close coupling of growth and reproduction to the annual increase in food supply during summer, apparently allow for a compensation of less productive winter periods to stabilize the high biomass and density of *L. elliptica* populations. Longer-term runoff conditions indicated to affect metabolism and feeding as well as to shift the individuals' energy balance and growth are particularly strong during summer. Hence an alteration of population dynamics and decreasing abundance of the species can be implicated under scenarios of rapidly increasing runoff exposures, i.e. runoff waves taking longer or carrying more sediment. There are currently only few field reports addressing sedimentation effects on populations of the Antarctic benthos. Chronic disturbance associated with turbid meltwater runoffs was reported to drastically diminishing biodiversity and abundance of the more sensitive benthic WAP community, including ascidians, whereas opportunistic deposit feeding polychaetes or the bivalve species *Y. eightsi* occurred more abundantly (Torre et al. 2012; Sicinski et al. 2012; Smale and Barnes 2008).

6.2.2 Ice scouring

Effects of ice scouring on *L. elliptica* populations most probably are much more direct. *L. elliptica* appears to be adapted to environments characterized by frequent moderate ice impacts due to its endobenthic ecotype, the ability to retract the siphon or to burrow back into sediments (manuscript IV). However, frequent ice impacts can lead to locally restricted scenarios of high mortality. This is induced by sediment movements, displacement of individuals from their burial locations, ice-induced injuries and/or subsequent predation. Deduced from the present study, particularly older age groups of a population will be affected by ice-scouring. Survival rates are much lower for older *L. elliptica* as demonstrated by both reduced burrowing activity and much higher mortality rates under injury compared to younger individuals (manuscript IV).

Overall reduced physiological rates such as metabolism, growth or meal processing times are indicated for several Antarctic benthic species including *L. elliptica* compared to ecologically equivalent species elsewhere (Peck 2005; Barnes and Peck 2008). This might implicate a generally reduced injury- or stress-regeneration capacity as well. However, there is evidence that cellular regeneration capacities of older individuals might be affected as stagnating rates of proliferation under ageing go hand in hand with increased mortality rates under injury (manuscript IV, **Additional Results**). If ice scouring causes shell- or tissue-damage, particularly older *L. elliptica* may not be able to effectively regenerate organismic integrity and might consequently be less able to survive frequent ice impact.

Compensation by fast individual regeneration and the overall growth of the population appears crucial in areas of frequent population-damage due to grounding ice. Hence population dynamics can shift to the dominant occurrence of comparably faster growing younger individuals, as already reported for other Antarctic benthic species such as the bivalve *Yoldia eightsi* or the bryozoan *Fenestrulina rugula* (Peck and Bullough 1993; Brown et al. 2004; Barnes and Clarke 2011). According to Smale and Barnes (2008), Antarctic benthic populations intensely disturbed by ice scouring can be constantly held at early successional stages, as the time span between disturbances is too short to allow a prolonged population development. Younger *L. elliptica* can, however, compensate consequences of moderate ice impact as seen in their increased capacity to burrow back as well as on lower mortality rates under injury. The higher burrowing speed may relate to body size and the fact that they live very much closer to the sediment surface and are more frequently subject to disturbance. Age-related burrowing activities enable younger individuals to selectively survive ice impacts and also indicate an all in all better physiological basic fitness, which contributes to a higher stress tolerance compared to older individuals. In younger age groups of an impacted *L. elliptica* population, however, frequent ice-scouring might go along with states of frequent population recovery, including individual physiological reorganisation and recolonisation by new recruitment of larvae from the water column. This, however, requires more investigation into parameters that address the population composition.

While a single ice scour is known to have an initial catastrophic impact on benthic communities longer-term consequences are characterized by a scenario of species recovery and/or return, depending on disturbance level and on time. A major effect apparently is a species-specifically affected population abundance as a recolonization of disturbed areas is depending on the species-specific characteristics of return and different taxa recover at different rates (Lee et al. 2001; Gutt 2001; Smale et al. 2008a; Barnes and Conlan 2007). This consequently leads to selection and dominant appearance of mobile pioneering species such as polychaetes or species such as *Yoldia eightsi*. Thus, under locally increasing ice scouring, abundance of sessile, slow growing invertebrates most likely will diminish as also indicated for *L. elliptica* investigated in the present study (manuscript IV, Smale 2008; Smale et al. 2008a)

6.2.3 Cumulative effects of sedimentation and ice scouring

Ahn (Ahn 1994) previously suggested that there is a trade-off between bottom substrate stability and food supply determining the vertical distribution of *L. elliptica*. Also the present

study gives evidence that superimposition of sublethal physiological consequences of long-term turbidity and directly lethal effects of ice scouring can contribute to diminishing population abundance and further shape the age structure. Under predicted environmental changes of the next decades, including medium-term (decades) increases in ice-scouring and longer-term (centuries) increases in sediment runoff (Fig. 1) these will most certainly set distribution limits of local *L. elliptica* populations. Effects on the Antarctic benthos will be particularly evident close to a glacier or ice-cliff. There is evidence for a nearshore vertical zonation effect depending on the gradient of runoff and ice scouring as well as a high patchiness (horizontal variability) across this depth-gradient promoted by drastic ice scours (for review see Smale and Barnes 2008; Turner et al. 2009).

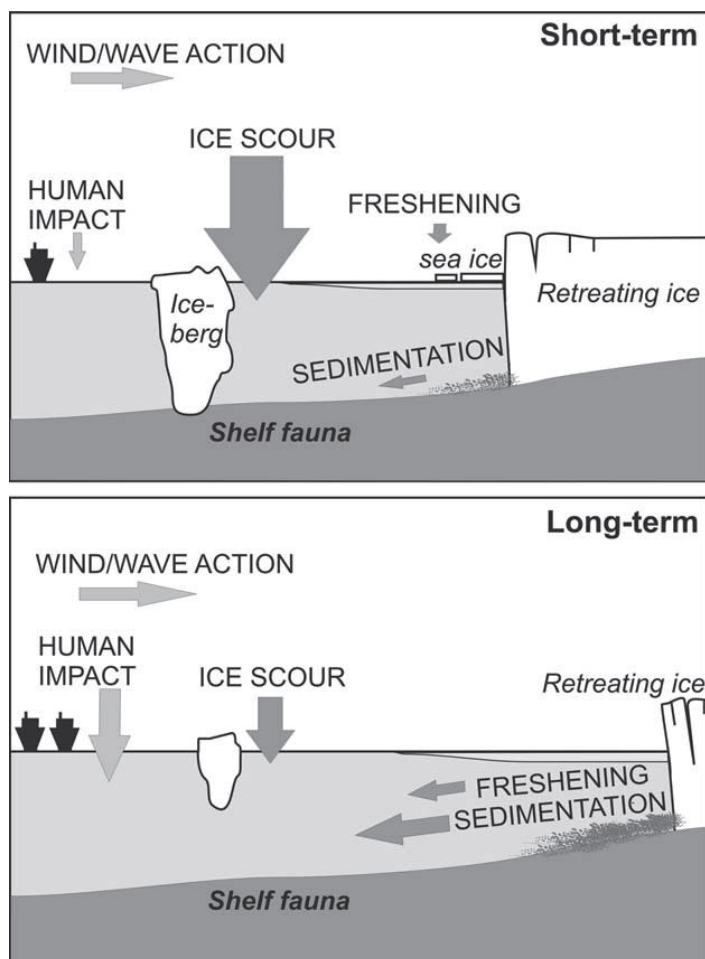


Figure 1: Importance of major physical disturbances influencing the benthic community structure in the WAP region. Likely future scenarios over both short (decades) and longer (centuries) timescales are shown. The size of the grey arrows indicate the relative importance of each major disturbance pressure taken from Smale and Barnes (2008).

6.3 Cellular and molecular stress- and immune response of *Laternula elliptica*

6.3.1 Specific characteristics of the *Laternula elliptica* immune functions

A major point of interest during this study was a basic characterization of *L. elliptica* innate immune functions with special emphasis on specific reactions of the circulating hemocytes towards external impact such as injury or microbial stimuli (manuscript **I** and **III**). *L. elliptica* displays similar components of innate immunity previously found in other bivalves. Free circulating hemocytes together with the hemolymph appear to be major mediators of immune defenses also in this bivalve species. Deduced from stimulation experiments using microbial stimulants (manuscript **II**), *L. elliptica* hemocytes have key functions in adsorption, immobilization, phagocytosis and intracellular destruction of microbes and smaller particles. A major task in the context of foreign particle recognition and phagocytosis is the generation of ROS and possibly RNS by hemocytes (“respiratory burst response”) (Lambeth 2004; Philipp et al. 2012b), which was also detected in *L. elliptica* hemocytes under microbial stimuli.

Alternatively, *L. elliptica* hemocytes are capable of spontaneously forming large aggregations for encapsulation and control of larger foreign particles or parasites. This response, according to Auffret (2005) and Donaghy et al. (2009b), is directly depending on membrane adherence mechanisms and linked to excretion of exudates. This is in line with *L. elliptica* hemocyte network formation under involvement of filopodia and excretion of exudates which was observed in the present study. Whether this capability contributes to an additional function in wound closure and repair remains to be clarified. Observations from the oyster *Pinctada fucata* or the slug *Limax maximus* indicated, that hemocytes additionally seem to be involved in wound healing not only by phagocytosis of damaged tissue but also in wound sealing by forming extracellular matrix components to avoid loss of hemolymph and microbial invasion (Acosta-Salmon and Southgate 2006; Ottaviani et al. 2010; Franchini and Ottaviani 2000).

Lower numbers of free floating hemocytes but higher basal and stimulated (zymosan) ROS generation per cell in younger animals indicated individual age to be an important modulator of the *L. elliptica* immune response. On the one hand age-related differences point to a decrease in the cellular ROS generation response capacity over lifetime. On the other hand these findings were accompanied by higher total hemocyte counts (THC) in older animals, pointing to an additional alteration of the hemolymph composition possibly made up by functionally different hemocyte types in older animals. A different composition of granular

and agranular hemocytes in younger and older individuals as well higher concentrations of granulocytes in older individuals was observed in the clam *Chamelea gallina* by Mosca et al. (Mosca et al. 2011). This suggested a better cellular immunocompetence (phagocytic activity) in older animals. Respiratory burst response and changes in THC are only two parameters of a concerted *L. elliptica* immune response capacity, therefore a concluding statement about age-related changes in the *L. elliptica* immunocompetence is not possible. It is not known in which way the amount of free floating hemocytes is modulated and to what extent different hemocyte numbers and/or cell types are located in the tissues. THC in *L. elliptica* is, however, modulated under environmental changes and e.g. after injury a significant amount of possibly former resident hemocytes appears to return to circulation if required (manuscript I).

Deduced from these results, the nature of the *L. elliptica* hemocyte immune response is depending on characteristics such as number, mobilization or turnover, intercellular recognition and most certainly type of hemocytes, as well as on the specific stressor (microbial invader). The nature of microbial recognition and adhesion appears to be essential in developing a specific immune reaction, as indicated by stimulus-specific morphological responses and stimulus-specific respiratory burst responses (manuscript I).

Based on RNA sequence analysis, further molecular components of the *L. elliptica* innate immune system include cellular receptors such as toll like receptors, cytokines (IL17), components of detoxification (cytochrome p450, metallothionein, ferritin), respiratory burst response and oxidative stress regulation (HIF, NOX, DUOX, alternative oxidase, SOD, catalase), protein stabilization (heat shock proteins) and cytoskeleton regeneration (e.g. TIMP) (for more details see manuscript III). These genes are important in the maintenance of cellular homeostasis under stressful conditions and enable *L. elliptica* to support an effective molecular response to external impact.

Similar to other bivalves (Canesi et al. 2002; Auffret 2005), immunity in *L. elliptica* apparently is additionally based on humoral factors such as antimicrobial peptides. Even though inhibition of bacterial growth by hemolymph and tissues was not observed in the present study, expression of a putative *L. elliptica* antimicrobial peptide, similar to *Hyriopsis cumingii* theromacin was verified throughout a set of different tissues and hemocytes. Further transcripts of putative antimicrobial peptides similar to *Mytilus* spp. mytilin-B, mytimycin as well as defensin were identified in the *L. elliptica* RNA sequence database. As little sequence and functional information exists for benthic invertebrates, in particular of Antarctic invertebrates such as *L. elliptica*, the annotated database of the present study provides an excellent resource for the discovery of putative genes involved in immunity and stress

response in *L. elliptica* and will further help to identify such genes in other bivalves (manuscript III).

6.3.2 Sensitivity of the *Laternula elliptica* cellular stress- and immune response to food restriction and injury

6.3.2.1 Cross effects of starvation and injury on the cellular immune response

Just as in most invertebrates lacking an adaptive immune system, a marker of *L. elliptica* immunomodulation apparently is a modulation of circulating hemocyte numbers demonstrating physiological changes inside the organism (for review see Auffret 2005). Observed changes in free floating hemocyte numbers (THC) in *L. elliptica* influenced by experimental changes of food supply or injury infliction as well as by experimental acclimatization pointed out THC is modulated under environmental impact and depending on the animals' physical state. Changes in THC are, however, rather general indicators of immunomodulation. These might go hand in hand with proportionally stronger effects on other hemolymph characteristics such as differential hemocyte composition, phagocytosis activity or ROS production. In the oyster *Crassostrea gigas* or the clam *Ruditapes decussatus*, such changes were already observed under laboratory conditions (Angel Hurtado et al. 2011). Although characteristics differing with age such as specific cellular ROS production were indicated by comparing different *L. elliptica* age classes in the present study, a modulation of ROS production, phagocytosis or hemocyte composition remains unclear under environmental immunomodulation.

Lower THC observed in starved compared to fed *L. elliptica* can be attributed to lower amounts of microbial stimuli and food particles in filtered seawater, which is in line with the downregulation of immunity-related genes in hemocytes of the same animals (manuscript III). Lowering THC under decreasing external stimuli as well as the direct upregulation of the amount of free floating hemocytes under acute stress therefore refer to functions in immune defenses digestion or transport (Oubella et al. 1994; Dang et al. 2011; Cochenec-Laureau et al. 2003). On the other hand, dramatically decreasing THC following laboratory acclimatization, lower THC under longer-term treatment with the absence of the animals natural sediments and the tendency of decreasing THC under absence of food point to a strong influence of the animals overall physiological state on THC levels.

Effects of starvation on immune functions in bivalves have rarely been investigated, however rather negative influences (e.g. lower hemocyte numbers or phagocytic activity) were detected in the oysters *C. gigas*, *C. virginica* or *Saccostrea glomerata* (Hegaret et al. 2004; Delaporte

et al. 2006; Butt et al. 2007). In insects (*D. melanogaster*) and also bivalves (*S. glomerata*) a link between high numbers of circulating hemocytes and also high resilience to microbial/parasitic impact was reported (Kacsoh and Schlenke 2012; Dang et al. 2011). This cannot functionally be proven when comparing similarly aged *L. elliptica* from the starvation and injury treatment. However, a constitutive turnover and maintenance of a high number of hemocytes is bound to be energetically costly for *L. elliptica*. Trends of decreasing THC under food restriction might indicate that trade offs exist between investment into a high stock of circulating hemocytes (such as energy allocation to hemocyte proliferation) versus investment in other aspects of fitness such as preservation of energy reserves. In *L. elliptica*, which appears highly adapted to extended periods of food restriction, this trade off might be important in order to ensure a response to acute stress if necessary.

This might be substantiated by an even higher responsiveness to acute stress (THC under injury) which was found in animals kept under the starvation regime, compared to fed animals, however, this remains speculative. It is not clear, if the increased number of circulating hemocytes originate from tissue migration or if these are primarily regulated by proliferation and cell death (such as hemocyte apoptosis (Terahara et al. 2005; Foster et al. 2011)). The overall capability for recruitment of the hemocyte stock under injury, however, was not reduced after several weeks of restricted food supply.

In spite of lower THC values, that were found in fed individuals under acute injury compared to the starved group, transcription levels of stress-response related genes appeared to be higher in hemocytes measured in the same individuals (manuscript **IV**). Thus, circulating hemocytes of fed specimens featured a different immune response compared to food restricted individuals characterized by higher induction of gene expression, whereas food restricted individuals started to recruit the hemocyte stock and increased hemocyte numbers after injury. Taken together, the energetic state of each individual considerably affects the characteristic and magnitude of the inducible stress response. However, cellular and also molecular responses to the starvation treatment were marginal. The majority of the investigated genes putatively involved in the *L. elliptica* stress response were neither significantly up- or downregulated in hemocytes or siphon tissues of the animals under starvation treatment, nor did starvation conditions systemically lead to a significantly altered expression response under additional stress (injury). In hemocytes, however, some clear effects on the up-regulation of these genes under injury compared with food restriction treatments highlighted the involvement in stress compensation of *L. elliptica* (manuscript **III**). On the one hand, effects of starvation in *L. elliptica* may not in the first line appear in hemocytes or siphon

tissues. However, there is evidence that indeed 3 or 6 weeks treatment time was not capable of effecting major starvation effects in *L. elliptica* as corroborated by gene expression analysis in digestive glands of the same animals. Altogether, the lack of strong responses under food restriction was ascribed to the adaptation of *L. elliptica* to the Antarctic environment including overall low metabolic rates and capability to survive under unfavorable environmental conditions. This was also shown for other Antarctic marine invertebrates (Peck 1998, 2002; Peck and Conway 2000) and is further discussed in the following section for *L. elliptica* (see also manuscript III).

6.3.2.2 Effect of short-term starvation on genes expression in digestive glands of *Laternula elliptica* under food restriction

Expression changes of selected genes most likely involved in metabolic response to food restriction represent only a small fraction of a comprehensive physiological response that is expected under changing environmental conditions. Nevertheless, the investigated candidate genes are not only important in keeping physiological integrity under varying external conditions but are also involved in physiological processes such as development or immunity. Although the effects of starvation in *L. elliptica* at an early stage might have been in particular apparent in digestive glands, only marginal changes in the overall gene expression under “starvation” treatment were detected. The digestive gland exhibits major functions of food digestion and storage of metabolic reserves in bivalves including *L. elliptica*, which can be utilized during periods of food restriction (Gosling 2003; Ahn et al. 2003).

The delay in the onset of severe starvation effects was substantiated by results of the hepatosomatic index (HSI) which is a measure of the animals condition and stored food reserves in fish and bivalves (Uehara and Shimizu 1999; Tankoua et al. 2012; Sabatini et al. 2011). Ahn et al. (2003) showed a significant reduction of *L. elliptica* HSI values during two Antarctic winters, which were directly correlated with total chlorophyll concentrations in the water column. In the present study, however, no consistent effects were attributed to the treatments “starvation” or “high food loads”, suggesting food reserves were not severely depleted (manuscript III).

The results can be ascribed to the adaptation of *L. elliptica* to the specific characteristics of the Antarctic environment. Antarctic invertebrates such as the brachiopod *Liothyrella uva* were shown to have typically prolonged post-prandial elevation of metabolic rates (Peck 1998, 2002; Peck and Veal 2001) which points towards a long duration of food utilization. The digestive gland in bivalves can contain a considerable amount of food material, as particle

flow and digestion of material takes place here (Gosling 2003). Together with prolonged food utilization-times, contents of prior uptaken food and stored reserve components might also have delayed an onset of severe starvation in *L. elliptica* used for the starvation experiment. Further Antarctic invertebrates typically have low metabolic rates as an adaptation to the extremely cold climate and intense seasonality with short summers and long periods of potential food shortage during winter (Clarke et al. 2008; Peck and Conway 2000; Schloss et al. 2012). According to Ahn and Shim (1998) overall metabolic rates of *L. elliptica* are well below those of temperate bivalve species and low metabolic rates even during summer appear to be an important strategy of the species energy conservation (see also reduced respiration under turbidity, manuscript IV). Therefore slow metabolic rates in *L. elliptica* might go hand in hand with non-sufficient treatment times to explain the lack in responsiveness under starvation treatments.

Nevertheless, a significant effect of food shortage on digestive gland gene expression was detected for *Le*-cyclin D (two-way ANOVA, small individuals). Cyclins such as Cyclin B and Cyclin D are regulators of the cell cycle. They were initially identified in the sea urchin *Arbacia punctulata* (Evans et al. 1983) and are functionally conserved between organisms, as shown also for bivalves (Swenson et al. 1986). Cyclin D is involved in G₁/S-phase-transition and starts to accumulate at mid-G₁-phase after extracellular growth signaling (Blagosklonny and Pardee 2002). In the snail *Littorina littorea* elevated levels of cyclin D mRNA expression in hemocytes were directly linked to an increase in hemocyte numbers and the onset of increased proliferation activity (Gorbushin and Iakovleva 2008). Downregulation of *Le*-cyclin D expression in digestive gland tissue of small *L. elliptica* specimens under food restriction is consistent with a similar (although non-significant) trend in large specimens of the same treatment. Moreover, transcript levels of *Le*-cyclin D were significantly (1.5-fold) higher accumulated in digestive glands collected after the end of the winter period compared with those collected during late summer. Together with the fact, that putative digestive enzymes (*Le*-cathepsin, see further down) were slightly upregulated at the same time, this might indicate that cellular proliferation of digestive gland tissues is closely linked to the punctual availability of food.

Another cellular mechanism, which is important in the context of cellular renewal and/or cellular reorganisation under external stimuli is autophagy. A putative *Le*-beclin1 in *L. elliptica* was identified due to high sequence similarities to Beclin1 sequences of other organisms such as *Xenopus tropicalis* or *Ixodes scapularis*. Beclin1 has a keyfunction to induce autophagy, which is an adaptive mechanism of degradation of cytoplasmic organelles

and proteins. *Le-beclin1* was expressed in all investigated treatments indicating a constitutive expression of the gene. This might emphasize the importance of a continuous Beclin1-mediated autophagy in developmental processes such as growth, cell-death or clearance from bacteria in order to allow tissue integrity (Cao and Klionsky 2007; Levine and Klionsky 2004; Godefroy et al. 2009). Also in the marine invertebrate *Ciona intestinalis*, autophagy was directly linked to the mRNA expression of Beclin during development (Godefroy et al. 2009). The slight up-regulation of *Le-beclin1* in both *L. elliptica* age-groups under starvation is in line with the evidence, that autophagy is inducible under periods of physiological stress to allow a survival of cells and/or tissues (Cao and Klionsky 2007; Tang et al. 2009). One of the major factors related to the pro-survival function of autophagy is caloric restriction. The induction and categorical need for autophagy under starvation has been demonstrated in various organisms including *S. cerevisiae*, *C. elegans*, *D. melanogaster*, *D. rerio* or *M. musculus* (Tsukada and Ohsumi 1993; Meléndez et al. 2003; Scott et al. 2004; Kuma et al. 2004; Yabu et al. 2012). The slight up-regulation of *Le-beclin1* in *L. elliptica* under starvation suggests an increasing need for keeping tissue integrity and cellular survival under food shortage.

Degradation/mobilization of cytoplasmic storage components might be in particular apparent in the digestive gland, to fuel the metabolism under starvation. In *L. elliptica* the major mobilization of energy reserves is based on protein metabolism (Ahn et al., 2003; Rodrigues et al., 2007). A major proteolytic enzyme with potential functions in intracellular degradation and development is cathepsin B as shown in amphioxus *Branchiostoma belcheri tsingtauense* or the invertebrates *Pandalus borealis* and *Meretrix meretrix* (Aoki et al. 2003; Wang et al. 2004; Yao et al. 2011). In response to starvation, gene expression of lysosomal proteolytic enzymes, such as cathepsin B can be induced in digestive glands, as shown also in clams *M. meretrix*. Here, levels of cathepsin B mRNA were significantly up-regulated under starvation and returned to basic levels when the clams were fed again (Yao et al. 2011). *Le-cathepsin B* mRNA levels were, however, slightly higher in the fed groups compared to the food restricted groups, possibly indicating a predominating function of cathepsin B in food digestion, rather than in mobilization of intracellular reserves under starvation.

This is substantiated by the finding, that the animals did not experience severe effects of starvation, and is further in keeping with non-significant effects on the expression of AMP activated protein kinase (*Le-AMPK*). AMPK is a key sensor of the cellular energy charge (ratio of AMP vs. ATP) and of cellular stress. The highly conserved enzyme is found in a variety of eukaryotes and plays a role in activating catabolic pathways and ATP generation in

mammals, while inactivating anabolic processes which are ATP-consuming (Pan and Hardie 2002; Hardie 2011). We found a high sequence similarity for the *Le*-AMPK transcript to AMPK sequences of other organisms. In marine crustaceans AMPK transcripts were differentially expressed in response to external stress such as heat-shock, hyperosmotic environments or starvation (Frederich et al. 2009; Zhu et al. 2007). The mechanism affecting the differential expression of AMPK under stress, however, needs further investigation, as starvation induced changes in functional presence of AMPK might be reflected by changes of enzymatic activity in addition to changes of transcriptional levels (Zhu et al. 2007; Lee et al. 2007). During starvation, AMPK transcript levels decreased in crustaceans *Artemia franciscana* (Zhu et al. 2007), which is in line with trends observed in *L. elliptica*. *Le*-amylase was chosen as it was expected to be a reliable marker for food consumption in *L. elliptica*. Gene expression of amylase in digestive tracts of marine bivalves such as *C. gigas* has been reported many times and was linked to digestion, growth and development (Prudence et al. 2006; Le Pennec and Le Pennec 2002; Huvet et al. 2008). Amylase-like molecules have been investigated in *C. gigas* and the marine mollusc *Haliotis discus discus*, where they were up-regulated according to food availability (Nikapitiya et al. 2009; Huvet et al. 2003). In contrast, no up-regulation of *Le*-amylase was detected in fed compared to starved *L. elliptica*, corroborating minor treatment effects and elevated food utilization times, and additionally possibly indicating a regulation of this enzyme, which is more on the level of enzymatic activity than on gene expression levels.

More prominent effects were observed for *Le*-ferritin. In invertebrates, ferritin is a major protein for iron transport and iron metabolism with potential functions in processes such as growth/proliferation (Missirlis et al. 2007; Li 2010; Huan et al. 2012) as well as immunity (Zhang et al. 2006; Perrigault and Allam 2012; Li et al. 2008; Simonsen et al. 2011). In bivalves (*Argopecten purpuratus*), up-regulated ferritin expression was ascribed to copper-detoxification (Zapata et al. 2009), whereas in *Pinctada fucata* and *Meretrix meretrix*, expression was additionally linked to development and shell formation (Zhang et al. 2003; Huan et al. 2012; Wang et al. 2009). Shell formation in bivalves is functionally located to the mantle, which continuously incorporates iron and other metals (Zhang et al. 2003; Marigomez et al. 2002). The putative *Le*-ferritin transcript in *L. elliptica* shares high sequence similarity with the sequence of the pearl oyster *P. fucata* (Zhang et al. 2003). While in *P. fucata* and also hard clams *Mercenaria mercenaria* highest ferritin mRNA expression was found in mantle tissue (Zhang et al. 2003; Perrigault and Allam 2012), *Le*-ferritin in *L. elliptica* was

prominently expressed in hemocytes, gills and digestive glands. Specific expression pattern might go hand in hand with tissue-specific functioning in metal metabolism.

Whereas for *P. fucata* a major involvement of ferritin in shell formation was proposed (Zhang et al. 2003), this had yet to be confirmed for *L. elliptica*. Also in *L. elliptica* iron incorporation into shells was reported, which was highest in young individuals (up to ~ 4 years), and significantly leveled off in older animals (Dick et al. 2007). In the present study, only tissues of older *L. elliptica* (~ 15 years old) were investigated for *Le*-ferritin expression and thus no conclusions of age dependend changes in ferritin expression in mantle tissue can be drawn. More interestingly, the multifold higher relative expression of *Le*-ferritin in gills, digestive glands and hemocytes compared to mantle tissue might reflect the physiological function of these components in bivalves and go hand in hand with the proposed way of *L. elliptica* metal accumulation from the environment (manuscript II). Gills constitute a major site of contact to ingested particulate matter and microbiota as well as uptake and intracellular incorporation of dissolved metals from the environment which then can be released to the hemolymph. High expression values of *Le*-ferritin mRNA in hemocytes are in line with a proposed key function in metal uptake, its transport and regulation (Marigomez et al. 2002; Missirlis et al. 2007).

The high expression of *Le*-ferritin mRNA in digestive glands is consistent with key roles of this organ in metal metabolism, metal storage, homeostatic regulation of the internal medium and secretion of xenobiotics. Metals are transported either from internal medium via the hemolymph plasma/hemocytes or from external via ingested particles to the digestive gland (Missirlis et al. 2007). Involvement of ferritin in intracellular trafficking of food associated iron is in line with slightly up-regulated expression levels of *Le*-ferritin found in fed compared to starved *L. elliptica* individuals. Similar to *Le*-cyclin D, this might also indicate that digestive gland metabolic rates and cellular development are initiated by the availability of food.

Animals from a metal enriched sedimentary environment most certainly have adapted to the high abundance of metals by corresponding detoxifying regulation mechanisms including expression of metal ligands similar to ferritin (this study) or metallothionein (this study, Choi et al. 2007; Park et al. 2007). Tissue-specific expression pattern found for *Le*-ferritin might further go hand in hand with the capability of *L. elliptica* to control uptake, regulation and excretion of metals from the environment.

Altogether results received from digestive glands are in line with major results from the hemocyte and siphon tissue expression analysis of candidate genes functionally related to immune- and general stress responses that were received from the same experimental animals

(manuscript **III**). There is evidence for a low stress response and slow metabolic response after several weeks of unfavorable feeding conditions pointing to the extraordinary capacity to survive the distinct seasonality of the Antarctic environment. Most prominent effects of food restriction were found in the expression of genes putatively involved in cellular development which might go hand in hand with a close coupling of growth to food supply.

6.3.2.3 Differences in stress response between younger and older *Laternula elliptica*

In a number of factors, age was a more important factor than the experimental treatment. In the cellular response to injury, the most prominent effects in transcriptional expression were detected in hemocytes of younger individuals following acute injury. On the one hand, this emphasises the functional role of hemocytes within the immune response of *L. elliptica* (manuscript **III**). On the other hand, a lack of strong effects in hemocytes of older specimens substantiates results of the cellular, biochemical and whole animals marker analysis of this study in which physiological state or stress response capacity varied with age (see **6.1** and **6.3.1**). The more prominent cellular up-regulation of stress response genes under injury in younger individuals shows that the induction period of a molecular stress response in young individuals might be shorter or younger animals might mount a more effective response with additional transcripts in order to counteract or mitigate potential cell damage under physiological stress. Physiological capacity to regenerate from injury might be higher in younger individuals as additionally pointed out by higher mantle proliferation rates (**Additional Results**), higher rates of annual shell growth (Philipp et al. 2008a) and general higher tissue protein turnover (Philipp et al. 2005a).

Hence a more flexible compensation response might contribute to a higher stress tolerance as seen by the much lower mortalities in younger animals under injury and faster decreasing general physiological condition in older individuals (manuscript **III** and **IV**). Altogether, data substantiate that younger *L. elliptica* might have a higher flexibility to react to environmental challenge, possibly pointing to a greater resilience and a better physiological condition.

It is not clear, however, if higher responsiveness and higher physiological rates that are indicated for younger specimens might contribute to a higher energetic dependency and possibly shift individuals under high stress frequencies away from energetic homeostasis and might on the long run contribute to overall affected lifetime somatic or gonad production (Sokolova et al. 2012; Brey et al. 2011).

Older specimens rather reduce metabolism under sediment stress and fail first when performing critical biological functions under ice impact such as reburrowing to the

sediments. This on the one hand is in line with a more sedentary lifestyle, manifested by deeper anchorage/burial to the sediments, growth of more robust shells and lower burrowing activity. On the other hand, on a cellular level, older specimens decrease growth and/or proliferation and can accumulate increasing amounts of cellular waste products. Together this might contribute to an age-dependency of physiological responses on different scales, a lower physiological flexibility and differing stress tolerance compared to younger species.

Lower proliferation rates in older animals can, however, not be generalized for marine bivalves, as the same observation was not made comparing juvenile and adult *M. galloprovincialis* (Zaldibar et al. 2008) and also not in all investigated tissues of *L. elliptica*, investigated in this study. Even when comparing extremely divergent age groups of the ocean quahog *A. islandica* (animals with an age of [A] 7-10 and of [B] 118-145 years), Strahl and Abele (2010) detected no significantly decreasing proliferation rates in different tissues such as mantle, gills and adductor muscle of this species with age (exception: heart tissue). Multifold lower proliferation rates, especially in the mantle epithelium of older compared to younger *L. elliptica* might indicate decreasing growth/cell turnover with age specifically in this species. Corresponding to the accordant age groups investigated in our study, the curve of the *L. elliptica* von Bertalanffy growth model calculated by Philipp et al. (2008a) was found to be almost exponential for younger animals with an age up to 10 years, meaning strong growth, and a stationary phase of down-slowning growth in animals up to 40 years. Reduced *L. elliptica* growth with age is in line with an increasing failure to remove oxidative stress-related protein damage with age (Philipp et al. 2005a; Philipp et al. 2008a). Moreover, the results of the present study from the comparison of stress markers in animals from the two distinctly disturbed environments point to an impact by turbid meltwaters or accumulation of metals from the environment which is rather long-term.

Altogether this might sequentially contribute to functional impairments with increasing age.

As stress-responses are integrating all levels of functional complexity (molecular, cellular, physiological) (Sokolova et al. 2012) this might have major implications for understanding a population response to environmental change. On the long run age-related susceptibilities to stress may not only lead to an alteration in the age structure of populations but also shift population dynamics. In *L. elliptica* gonad development rapidly increases with shell length (age) and older individuals around 80 mm shell length appear to have the highest reproductive output and hence are more important for population reproduction (Kang et al. 2003; Urban and Mercuri 1998). A shift towards younger/smaller individuals might have major implications for future population response under environmental perturbation and might not

only influence population dynamics of key suspension feeders *L. elliptica* but also trigger a cascade of secondary extinctions, altering the composition and function of Antarctic shallow benthic communities. Predicted effects on *L. elliptica* population age structure and abundance will shape nearshore ecosystems of the WAP as *L. elliptica* has a significant effect on the biodeposition from the pelagial to the benthos.

SUMMARY

Increasing temperatures and glacier-disintegration at the Western Antarctic Peninsula (WAP) are presently altering environmental conditions in shallow coastal areas. Rising water temperatures, enhanced ice scouring impacts as well as increasing input of inorganic sediments from melt water runoff are anticipated to particularly affect slow growing sessile benthic filter feeders like the Antarctic soft shell clam *Laternula elliptica*, a long-lived species which is a major component of the nearshore benthic infauna.

In order to address the question of how populations will be affected by current and future changes of the nearshore environment, this thesis set out to investigate a suite of whole organismic, biochemical, cellular and molecular responses of *L. elliptica* under effects of increased turbidity, ice scouring and food restriction particularly focusing on age-specific sensitivities.

Behaviour, metabolism, cellular accumulation of metals as well as oxidative stress markers were investigated in young and old individuals from two field-stations of the Potter Cove (King George Island/Isla 25 de Mayo, WAP), which are characterized by different scales of turbidity or ice scouring.

Comparison of age-related responses pointed out younger individuals coped better with high turbidity, as seen on their stable respiration rates and were able to selectively survive effects of ice scouring, as indicated by their higher reburrowing activity and higher survival rates after injury, compared to older individuals. Also cellular products of oxidative stress (lipofuscin) and most metals investigated were found to be age-related and accumulate over lifetime whereas higher sedimentation or physiological stress due to ice scouring could not consistently be related to these markers (lipofuscin, protein carbonyls, metals). Tissue trace metal content rather seems to originate from the water column or food source than from sedimentation per se. The comparison of animals from both field stations suggest that inorganic turbidity and ice-impact close to a glacier might affect lifetime growth, contribute to a decreasing physiological state in older animals which may lead to a selective survival of younger individuals finally decreasing populations lifespan and abundance.

To further understand basic cellular functioning of the *L. elliptica* stress and immune defense, hemocyte (immunocytes) responses under physical stress and microbial exposure were investigated. On the one hand cellular responses to microbial stimuli were characterized *in vitro*, on the other hand food restriction and injury experiments were undertaken using different age classes of *L. elliptica*, in order to study age-dependent *in vivo* responses of

cellular immunity (abundance of free circulating hemocytes). Hemocytes of *L. elliptica* featured common immune functions such as adsorption, immobilization, phagocytosis of microbes and particles, as well as the generation of reactive oxygen species (ROS). Younger clams had lower numbers of hemocytes but higher basal and stimulated (zymosan) ROS generation per cell compared to older clams, indicating changes in immune response capacities over lifetime. Trends of increasing numbers of free floating hemocytes under extrinsic factors such as experimentally increased food supply or injury showed that characteristics of hemocyte mediated immune responses in *L. elliptica* are influenced by the animals' physical state and subject to environmental changes.

For the molecular investigation of the *L. elliptica* stress response a mRNA sequence dataset was generated from hemocytes and tissues of stress challenged (injury, food restriction) individuals using 454 sequencing. The annotated dataset was searched for genes with putative functions in immune and stress responses as well as metabolism. Expression changes of selected candidate genes were investigated in hemocytes, siphon and digestive gland of young and old individuals subjected to food restriction and injury treatments. Selected genes were rather affected by injury than by starvation, but the effect of injury was modulated by the nutrition treatment. Particularly in hemocytes of younger, fed individuals, expression levels increased after acute injury, whereas in young starved or old individuals only minor expression changes could be observed. The stress response of *L. elliptica* thus depends not only on the nature of the environmental cue but particularly on age.

Low effects of food-restriction on physiological state and gene expression were ascribed to the species adaptation to characteristics of the Antarctic environment including low metabolic rates and might go hand in hand with the enhanced capability to survive under unfavourable environmental conditions. The overall results indicate that the physiological state and stress response of *L. elliptica* is connected with environmental stress levels but is even higher related to the individuals' age. Age related differences in burrowing activity, tissue proliferation rates, oxidative stress and stress response may lead to specific survival of younger individuals under the ongoing changes at the WAP coastal ecosystems thus affecting *L. elliptica* population age demographics and abundance and with this the benthic ecosystem this key-species is living in.

ZUSAMMENFASSUNG

Steigende atmosphärische Temperaturen sowie ein massives Abschmelzen lokaler Gletscher führen in küstennahen Schelfgebieten der westantarktischen Halbinsel (WAP) zu komplexen Veränderungen der Umweltbedingungen. Gerade langsam wachsende, sessile Filtrierer wie die antarktische Schlickmuschel *Laternula elliptica*, könnten durch einen hierdurch bedingten Anstieg der Wassertemperaturen, vermehrten Eintrag anorganischer Sedimente sowie zunehmende Störung durch flottierende Eismassen betroffen sein. *L. elliptica* ist ein langlebiger Vertreter des Makrobenthos, der zirkumpolar verbreitet ist und in einigen Bereichen der WAP das küstennahe Endobenthos dominiert.

In der vorliegenden Arbeit sollten sowohl organismische, biochemische, zelluläre als auch molekulare Untersuchungen Aufschluss über die physiologische Reaktion von *L. elliptica* Individuen auf mögliche Stressfaktoren im Bereich eines zurückgehenden Gletschers geben. Die Untersuchung unterschiedlich alter Individuen sollte Effekte auf die physiologische Fitness und das Überleben verschiedener Altersgruppen erfassen.

Im ersten Teil der Arbeit wurden zwei Subpopulationen von *L. elliptica* beprobt, die in der Potter Cove (King George Island/Isla 25 de Mayo, WAP) sowohl den Stressfaktoren Sedimenteinfluss als auch Eisgang unterschiedlich stark ausgesetzt sind. Anhand von Markern bezüglich des Verhaltens, des Metabolismus, der zellulären Anreicherung von Metallen aus dem Habitat, sowie des zellulären oxidativen Stresses sollte der physiologische *in situ*-Status dieser beiden Subpopulationen erfasst werden. Hierbei zeichnete sich bei einem Vergleich der Altersgruppen ab, dass jüngere *L. elliptica* im Gegensatz zu den älteren Individuen unter starker mineralischer Trübung stabile Respirationsraten aufrechterhalten konnten. Vielfach höhere Überlebensraten nach Verletzungen, sowie ausgeprägtere Fähigkeiten, nach der Störung durch strandendes Eis in den Boden zurückzukehren, deuteten zusätzlich daraufhin, dass jüngere Individuen eher in der Lage sein könnten, diese Störungen selektiv zu überstehen.

Eine Altersabhängigkeit wurde auch auf der Ebene der Konzentration zellulärer Marker gezeigt, wobei im Fall von Lipofuszin, einem Marker oxidativen Stresses, sowie der meisten untersuchten Metalle mit dem Alter eine Zunahme beobachtet wurde. Innerhalb der stärker beeinflussten Subpopulation konnten dem direkten Einfluss des abschmelzenden Gletschers keine einheitlichen Effekte auf zelluläre Marker oxidativen Stresses (Lipofuszin, Proteincarbonyle) zugeschrieben werden. Auch führte in *L. elliptica* die direkte Nähe zu den Hauptabflüssen von Schmelzwasser und erodierter vulkanischer Sedimente nicht zu einer

Gewebe-Anreicherung von Metallen aus dem Habitat. Ein bestimmender Faktor war hier stattdessen wiederum das Alter der Tiere. Auch scheint die Metallanreicherung innerhalb der untersuchten Gewebe eher aus der Wassersäule oder aus der Nahrung zu stammen und nicht durch Sedimentaufnahme bedingt zu sein. Insgesamt deuteten diese Ergebnisse daraufhin, dass ein Sedimentabfluss vom Gletscher kaum zu kurzfristigen Veränderungen in der Metallakkumulierung führt, sich andererseits aber langfristig auf Parameter wie die Wachstumsrate oder die Gesamtbilanz physiologischen Stresses (Lipofuszin) auswirken könnte. Ein verschlechterter physiologischer Zustand, der sich in älteren Tieren zeigte, könnte in Gebieten starker Gletscherabflüsse und gleichzeitig hohem Störungsaufkommen durch strandende Eis-Massen insgesamt zu einer Veränderung der Populations-Demographie und gleichzeitig rückgängigen Abundanzen beitragen.

Um im Kontext rapider Umweltveränderungen zelluläre Stressreaktionen von *L. elliptica* besser einschätzen zu können, wurden im zweiten Teil der Arbeit basale Parameter der Stress- und Immun-Antwort charakterisiert, wobei der Fokus auf den zirkulierenden Immunocyten (Hämocyten) lag. Hierzu wurden einerseits die zelluläre Reaktionen auf mikrobielle Stimulation *in vitro* charakterisiert, andererseits wurden verschieden alte Tiere experimentell unter Nahrungsmangel sowie künstlichen Verletzungen gehalten, um eine mögliche zelluläre Antwort *in vivo* zu charakterisieren. Hämocyten von *L. elliptica* zeigten generelle Funktionen wie Adsorption, Immobilisierung und Phagozytose von Bakterien und Partikeln, sowie Generierung von reaktiven Sauerstoffradikalen (ROS). Spezifische Marker der Evertebraten-Immunität (Hämocyten-Konzentration und ROS-Generierung) waren altersabhängig. Jüngere Individuen wiesen eine geringere Hämocyten-Konzentrationen, jedoch eine höhere ROS-Produktion pro Zelle (stimuliert und unstimuliert) gegenüber älteren Individuen auf. Ein erhöhtes Nahrungsangebot sowie künstlich herbeigeführte Verletzung führte zu einer Zunahme der Hämocyten-Konzentration innerhalb der Hämolymphe. Dies deutete auf einen direkten Einfluss externer Faktoren wie Nahrungsangebot auf Immunparameter dieser Spezies hin.

Für die Charakterisierung molekularer Stress-Reaktionen wurde mittels 454-Sequenzierung eine mRNA Sequenz-Datenbank erstellt. Dafür wurde sowohl RNA aus Hämocyten als auch aus verschiedenen Geweben künstlich verletzter und gehungerter *L. elliptica* verwendet. Die Analyse der Expression von Genen, die aufgrund ihrer Homologie zu funktionell stress-kompensatorischen Genen anderer Organismen identifiziert werden konnten, deutete ebenfalls auf eine Altersabhängigkeit der Stressreaktion hin. Eine signifikante Hochregulierung zeigte sich nur in den Hämocyten verletzter Individuen der jüngeren

Altersgruppe, wobei diese Reaktion vom experimentellen Nahrungsangebot abhängig war. Im Gegensatz hierzu konnte weder in den Hämocyten oder im Siphogewebe älterer Tiere, eine differentielle Expression nach Verletzung nachgewiesen werden. Auch konnten in den Mitteldarmdrüsen gehungerter Tiere kaum relevante Expressionsänderungen von metabolischen Genen nachgewiesen werden. Die geringen Effekte experimenteller Nahrungsknappheit auf Physiologie und Genexpression in *L. elliptica* könnten mit der evolutionären Anpassung an die spezifischen Umweltbedingungen der maritimen Antarktis erklärt werden, die durch Reduktion basaler Stoffwechselfunktionen geprägt ist, um somit ein Überleben zu sichern.

Die Ergebnisse der vorliegenden Arbeit deuten daraufhin, dass sowohl physiologische Parameter wie Wachstumsraten oder oxidativer Stress, als auch physiologische Stressreaktionen von *L. elliptica* durch externe Stressfaktoren wie starke Trübung durch inorganische Sedimente oder strandendes Eis beeinflusst werden, wobei diese Faktoren wesentlich größeren Ausmaßes vom individuellen Alter geprägt sind. Ein höheres Stresskompensationspotenzial jüngerer Individuen könnte zum selektiven Überleben jüngerer Altersgruppen und somit zu einer Veränderung der Altersstruktur in Populationen führen, die vom Wandel der Umweltbedingungen in Flachwasserökosystemen besonders betroffen sind.

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