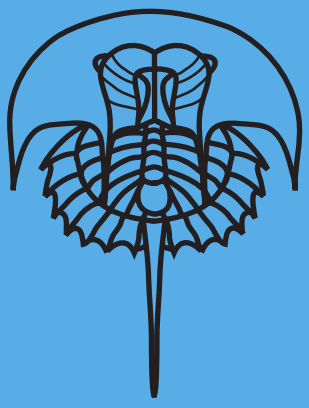

TREATISE ONLINE

Number 92

Part N, Revised, Volume 1, Chapter 7: Ecophysiology of Extant Marine Bivalvia

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2017

KU PALEONTOLOGICAL INSTITUTE
The University of Kansas

Lawrence, Kansas, USA
ISSN 2153-4012
paleo.ku.edu/treatiseonline

PART N, REVISED, VOLUME 1, CHAPTER 7: ECOPHYSIOLOGY OF EXTANT MARINE BIVALVIA

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SIZE AND COMPOSITION OF BIVALVE BODY

BODY SIZE

Most bivalves start their life as small pelagic larvae (i.e., trochophora and veliger stages) that have attained a size of <0.4 mm at the time of metamorphosis and settlement on the sea floor (Bayne, 1983; Raby & others, 1997). In species with parental care, particularly in brooding taxa such as the Corbiculidae, Pisidiidae, and Philobryidae, the offspring starts its independent life as a much larger juvenile. However, size at release is species specific and may correspond to environmental harshness and evolutionary history (e.g., Morton, 1978; Richardson, 1979; Brey & Hain, 1992; Glaubrecht, Fehér, & Von Rintelen, 2006; Mackie, 2007). Fresh-water Unionida represent a special case in which the glochidia larvae attach to gills or skin of a host fish (Bauer & Wächtler, 2001).

In terms of the maximum and minimum (recorded) body size, extant bivalves cover more than 3 orders of magnitude. The smallest bivalve species known so far is the nut clam *Condylonucula maya* Moore, 1977 (Nuculidae, Nuculoidea) that grows to a length of about 0.54 mm (Moore, 1977; Bouchet & Huber, 2015). The largest body size has been reported in the tropical *Tridacna gigas* (Linnaeus, 1758 in 1758–1759), the so-called giant clam (Cardioidea), that can attain up to 130 cm in length (Poutiers, 1998) and a mass of 500 kg (Griffiths & Klumpp, 1996; Adulyanukosol, 1997).

BODY COMPOSITION

A simple way to look at bivalve body composition is to separate it into shell, water, and dry soft parts. According to the conversion-factor database of Brey and others (2010), available from Brey (2001), the average bivalve consists of 62.0% shell, 33.5% water, and 6.7% dry body mass (calculated from the factors given in Table 1). In the following discussion of shell and soft-body composition, we refer to Brey and others (2010) and Eleftheriou (2013) for definition and determination of major mass units: i.e., wet mass including shell (WM + Shell), wet mass (WM, without shell), dry mass (DM, without shell), and ash-free dry mass (AFDM, without shell).

BIVALVE SHELL

The bivalve shell is a complex composite of mineralized calcium carbonate ($CaCO_3$) and organic components. Most shells are composed of the two carbonate polymorphs, aragonite and calcite, but in some taxa the $CaCO_3$ mineral phase is clearly dominated by either calcite (e.g., Ostreida, Pectinoidea) or aragonite (e.g., Arcticidae, Unionidae) (Taylor, Kennedy, & Hall, 1969, 1973; Carter, 1980; Chateigner, Morales, & Harper, 2002). Very rarely a third polymorph, vaterite, is present—for example,

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TABLE 1. Share of important components in total body mass (ratios). See ELEFTHERIOU (2013) for further details on determination of body mass and energy content. *AFDM,* ash-free dry mass (*DM* – ash remaining after incineration); *DM,* dry mass; *J,* joule; *N,* number of samples; *WM,* wet mass; *WM+Shell,* wet mass, including shell mass. Data derived from Conversion04 database (BREY & others, 2010).

	Mean	Standard deviation	Median	Minimum	Maximum	Number of samples
WM/(WM+Shell)	0.379	0.198	0.36	0.031	0.7	19
DM/WM	0.177	0.078	0.173	0.035	0.5	81
AFDM/DM	0.833	0.091	0.852	0.495	0.994	164
AFDM/WM	0.139	0.063	0.129	0.027	0.29	54
Protein/DM	0.484	0.117	0.486	0.179	0.697	60
Carbohydrate/DM	0.203	0.123	0.195	0.02	0.615	51
Lipid/DM	0.087	0.038	0.082	0.027	0.235	60
C_{org}/DM	0.35	0.064	0.35	0.227	0.454	15
N/DM	0.086	0.017	0.086	0.033	0.138	32
C/N	4.159	1.18	3.985	2.529	6.984	14
J/mgWM	3.178	1.403	3.135	0.675	5.94	51
J/mgDM	17.95	2.474	18.072	10.077	25.534	186
J/mgAFDM	21.38	2.43	21.395	12.796	29.082	149

in the freshwater *Corbicula fluminea* (O. F. MÜLLER, 1774 in 1773–1774) (see SPANN, HARPER, & ALDRIDGE, 2010) and in the marine mud clam *Laternula elliptica* (KING, 1832 in KING & BRODERIP, 1832) from Antarctica (NEHRKE & others, 2012). Furthermore, amorphous $CaCO_3$ (ACC) plays a role as a precursor phase in the mineralization process (WATANABE, 1983; WEISS & others, 2002; RADHA & others, 2010).

The organic matrix of the shell usually constitutes just a few percent of total shell mass. Depending on taxon and age, this organic share ranges between 0.05% and 5.3% of shell mass (KIDWELL, 2005). The chemical composition of this matrix is very complex and diverse (KOBAYASHI & SAMATA, 2006), primarily consisting of glucoproteins proteins, and beta-chitin (LEVI-KALISMAN & others, 2001), but also containing lipids (FARRE & DAUPHIN, 2009). Some components of the organic matrix play a role in the mineralization process (WEINER, 1983; CUSACK & FREER, 2008), whereas others contribute to the strength of the shell structure (OKUMURA & DE GENNES, 2001). The specific function of many components, however, remains enigmatic. For instance, polyenes contribute to shell coloration (HEDEGAARD, BARDEAU, & CHATEIGNER, 2006), but it remains unclear whether this is their primary and only function, as they can be found in the shells of species that lack coloration, such as *Arctica islandica* (LINNAEUS, 1767 in 1766–1767) (STEMMER & NEHRKE, 2014). For detailed information on shell structure and biomineralization, see LOWENSTAM and WEINER (1989), CARTER (1980), WEINER and DOVE (2003), ADDADI and others (2006), JACOB and others (2008), MUELLER (2011), and MARIN, LE ROY, and MARIE (2012).

BIVALVE SOFT BODY

There is no indication that the soft-body composition of bivalves deviates distinctly from the average aquatic benthic invertebrate. As summarized in Table 1, the ratios of protein/dry mass (0.484), carbohydrate/dry mass (0.203), lipid/DM (0.087), and ash/DM (0.167) add up to 0.941 (i.e., almost 1.0), which is good evidence that these data are reliable. Moreover, the average energy content values in Table 1, derived from BREY and others (2010), confirm earlier compilations

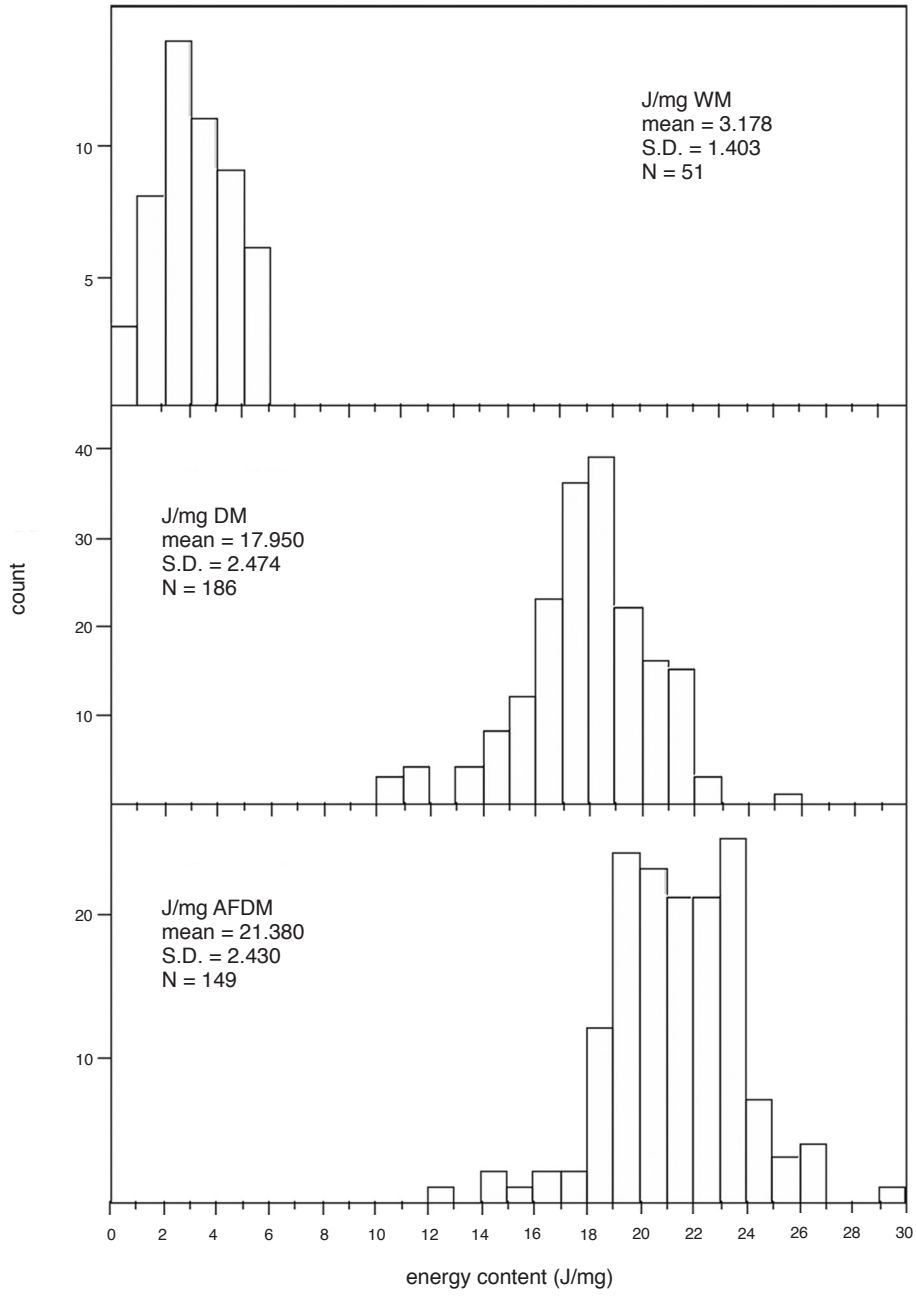

FIG. 1. Energy content (J/mg) in bivalve wet mass (WM), dry mass (DM), and ash-free dry mass (AFDM); data from Conversion04 database (BREY & others, 2010) (new).

(BEUKEMA, 1997). The bivalve values are within the range observed across all aquatic animals: 3.525 ± 1.985 J/mg WM, 16.020 ± 5.175 J/mg DM, and 22.184 ± 2.906 J/mg AFDM (BREY & others, 2010). The minimum and maximum values of the observed range (Fig. 1), however, should not be overinterpreted, as they may be subject to methodical errors.

VARIABILITY IN BODY COMPOSITION

Bivalve body composition can be affected by developmental stage or age, by the reproductive cycle (building and release of gonad products), and by environmentally driven seasonal cycles in body growth and investment in storage components. Many studies indicate a complex interplay of these factors that may even cause different organs to display differing temporal patterns in their composition. Studies on a variety of bivalve species—e.g., the venerid clam *Eurhomalea exalbida* (Dillwyn, 1817) from Patagonia (Lomovasky, Malanga, & Calvo, 2004), the oyster *Hyotissa hyotis* (Linnaeus, 1758 in 1758–1759) from the Gulf of California (Rodriguez-Astudillo & others, 2005), or the mud clam *Laternula elliptica* from Antarctica (Ahn & others, 2003)—indicate some general patterns. These include (1) proteins serving as an important energy-storage component, mainly in muscle tissues, that are built up under favorable conditions of alimentation and used for somatic growth, gonad production, and energy during times of food shortage (winter); and (2) lipid and carbohydrate levels being closely coupled with the reproductive cycle.

The significance of age for body composition is less well understood. Sukhotin and Flyachinskaya (2009) showed that in the blue mussel *Mytilus edulis* Linnaeus, 1758 in 1758–1759, the share of gonad tissue in total body mass (and, thus, the individual fecundity) decreased significantly with age, indicating reproductive senescence. The same tendency towards reproductive senescence and a preference for somatic growth in aging individuals is also found in the short-lived group of scallops. Especially in fast-growing species such as *Argopecten irradians irradians* (Lamarck, 1819 in 1818–1822) with a lifespan of 2–3 years, but also in *Chlamys islandica* (Müller, 1776) with a 20-year lifespan, weight-specific reproductive effort declines significantly in the second year, as in *A. irradians irradians,* or at the end of lifespan, as *C. islandica* (Bricelj & Krause, 1992). On the contrary, in the tropical Catarina clam, *A. ventricosus* (G. B. Sowerby II, 1842 in Sowerby II & others, 1842–1887), a constant gonadosomatic index (GI), calculated as the percentage of gonad to empty shell weight, in first- and second-year individuals of the same cohort indicated the investment into gonadal growth to be constant over the short lifetime of the species (Guerra, Zenteno-Savín, & others, 2012). The unaltered GI was, however, achieved at the expense of a decline in tissue maintenance, visible in an accumulation of the aging and stress-marker lipofuscin (the so-called fluorescent age pigments) in somatic tissues during the second year. In extremely long-lived bivalve populations around Iceland of the ocean quahog, *Arctica islandica*, undiminished cellular maintenance (Strahl & Abele, 2010) and reproduction (compiled in Abele & Philipp, 2012) can be observed over most of the lifetime of the nominal species.

ENVIRONMENTAL INFLUENCES ON SHELL FORMATION

There are four major environmental constraints of shell formation: concentration of calcium ions (Ca^{2+}) in the water, concentration of carbonate ions (CO_3^{2-}) in the water, water temperature, and food availability. As discussed below, many studies have demonstrated the effects of these parameters on bivalve shell formation. However, we still lack a full understanding of the shell-formation process in bivalves, including the specific role of mantle epithelium and extrapallial fluid in initiation and control of crystal formation (Addadi & others, 2006; Nudelman & others, 2007). Therefore, science is still in the process of moving from empirical observation to a full cause-and-effect based mechanistic understanding of shell formation.

TEMPERATURE AND FOOD AVAILABILITY

Temperature is a major determinant of metabolic activity in poikilothermic organisms. Within the organism's temperature tolerance window, metabolism and such derivative functions as growth rates increase exponentially with temperature (rate = $e^{-c/T}$, where e is the base, c is a constant, and T is temperature)(Brown & others, 2004; Brey & others, 2010). Shell growth is a biologically controlled process, and hence the dynamics of the organic shell matrix are subject to the same temperature constraints as body growth. Moreover, temperature plays a role in the carbonate system, as discussed below.

Food availability is an equally important constraint. A bivalve will concentrate energy investments on basic maintenance and scale back on investments in growth of soft body and shell when food becomes scarce. However, the response depends on the species and specific temperature and food-supply conditions (Pilditch & Grant, 1999; Carmichael, Shriver, & Valiela, 2004; Melzner & others, 2011). Hence, temperature and food availability are presumed to be major determinants of seasonal cycles in shell growth of bivalves from temperate and polar regions, causing the formation of distinct shell growth increments in many species: e.g., *Arctica islandica* (Schöne & others, 2005), *Clinocardium ciliatum* (O. Fabricius, 1780); *Pecten maximus* (Linnaeus, 1758 in 1758–1759); *Laternula elliptica* (Brey & Mackensen, 1997; Chauvaud & others, 2005; Sejr, Blicher, & Rysgaard, 2009).

CALCIUM CARBONATE DYNAMICS

The boundary conditions for the formation of calcium carbonate ($Ca^{2+} + CO_3^{2-} \rightleftharpoons CaCO_3$) are defined by the saturation state of the water (Ω):

$$\Omega = [Ca^{2+}][CO_3^{2-}]/K^*_{sp},$$

where K^*_{sp} is the stoichiometric solubility product of the concentrations at equilibrium. For aragonite and calcite, K^*_{sp} is $10^{-6.19}$ and $10^{-6.37}$ mol^2kg^{-2}, respectively, under similar conditions (25°C, salinity of 35, and 1 atmosphere (atm) of pressure; for further details, see Zeebe & Wolf-Gladrow, 2001). Hence, $\Omega>1$ indicates supersaturation, or favorable conditions for $CaCO_3$ mineralization, whereas $\Omega<1$ indicates conditions in which carbonates tend to dissolve. Calcium-carbonate shell formation and maintenance becomes more difficult and energetically costly with decreasing Ω. Based on the assumption that the energetic costs of shell formation in shallow-water marine mollusks are in the range of 1–2 J/mg $CaCO_3$ (Palmer, 1992), these additional costs may constitute a significant drain on the animal's energy budget. The equilibrium constants indicate that aragonitic shells may dissolve more readily than calcitic ones. Experiments, however, show that factors such as crystal size and organic matrix structure affect dissolution processes more significantly than mineralogy (Harper, 2000).

The calcium-ion concentration correlates with salinity: 0.4 g kg^{-1} at a salinity of 35 to <0.1 g kg^{-1} in fresh water (Johnson, Liquorish, & Sha, 2007). The carbonate-ion concentration at saturation, however, increases slightly with decreasing temperature (<1% change in calcite and <5% in aragonite); it increases with salinity—from 9.9 or 6.9 µmol kg^{-1} in fresh water (25 mg Ca^{2+} kg^{-1}) to 41.9 or 67.0 µmol kg^{-1} (salinity of 35, at 1 atm, 2°C) for calcite and aragonite, respectively; and it also increases with pressure—from 41.9 or 67 µmol kg^{-1} (1 atm, 2°C) to 111.7 or 167 µmol kg^{-1} (500 atm, 2°C) for calcite and aragonite, respectively (Plummer & Busenberg, 1982; Zeebe & Wolf-Gladrow, 2001; D. Wolf-Gladrow, 2015, personal communication).

Correspondingly, at given concentrations of Ca^{2+} and CO_3^{2-}, calcium-carbonate shell formation and maintenance becomes more difficult and costly with decreasing temperature, with decreasing salinity, and with increasing pressure. Hence, conditions for bivalve

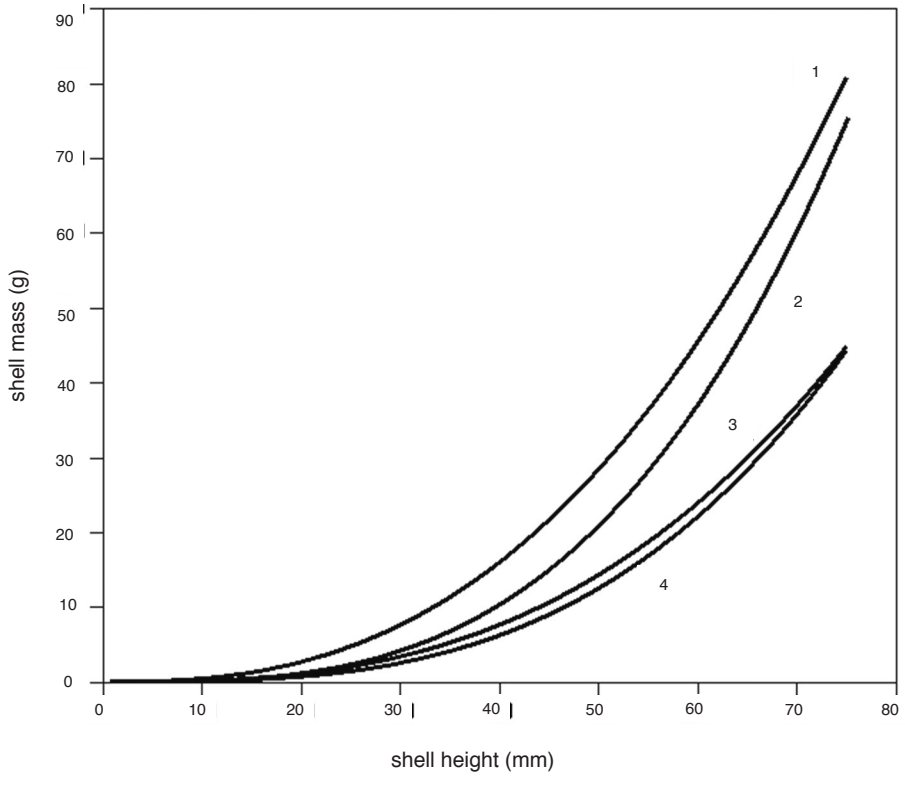

FIG. 2. The relationship of shell mass to shell height in *Arctica islandica* (LINNAEUS, 1767 in 1766–1767) from different sites: *1,* Norwegian Coast, salinity = 33); *2,* Iceland and German Bight, salinity = 35 and 31; *3,* Kattegatt and White Sea, salinity = 30 and 25); *4,* Kiel Bight, salinity = 25 (Begum & others, 2010).

shell formation change distinctly along the two major aquatic environmental gradients, water depth (i.e., pressure) and salinity.

Surface ocean waters today are supersaturated in CO_3^{2-} for both calcite and aragonite shell formation. Owing to increasing pressure, Ω decreases with water depth, and at a certain water depth in situ carbonate concentrations fall below saturation concentrations (Ω becomes <1). Below this "saturation depth" (between 500 and 5000 meters, depending on ocean, latitude and $CaCO_3$ polymorph) shells must be protected from dissolution, and dead shells will dissolve over time. Regrettably, there are no systematic studies that relate bivalve shell thickness or periostracum (organic shell cover) strength to water depth.

Concentrations of calcium and carbonate ions decrease from fully saline to fresh water environments, and apparently this has an effect on bivalve shell thickness. Thin-shelled species predominate in fresh waters, whereas thick-shelled taxa (e.g., hard clams) are restricted to the marine realm. A decrease in shell thickness and shell mass is also seen within a species along the salinity gradient from fully marine to brackish waters—for example, in the ocean quahog *Arctica islandica* (calculated from BEGUM & others, 2010; Fig. 2).

CO_2 AND OCEAN ACIDIFICATION

The following description of the basic properties of the carbonate system closely follows the excellent textbook of ZEEBE and WOLF-GLADROW (2001). The carbonate-ion concentration (CO_3^{2-}) depends on the

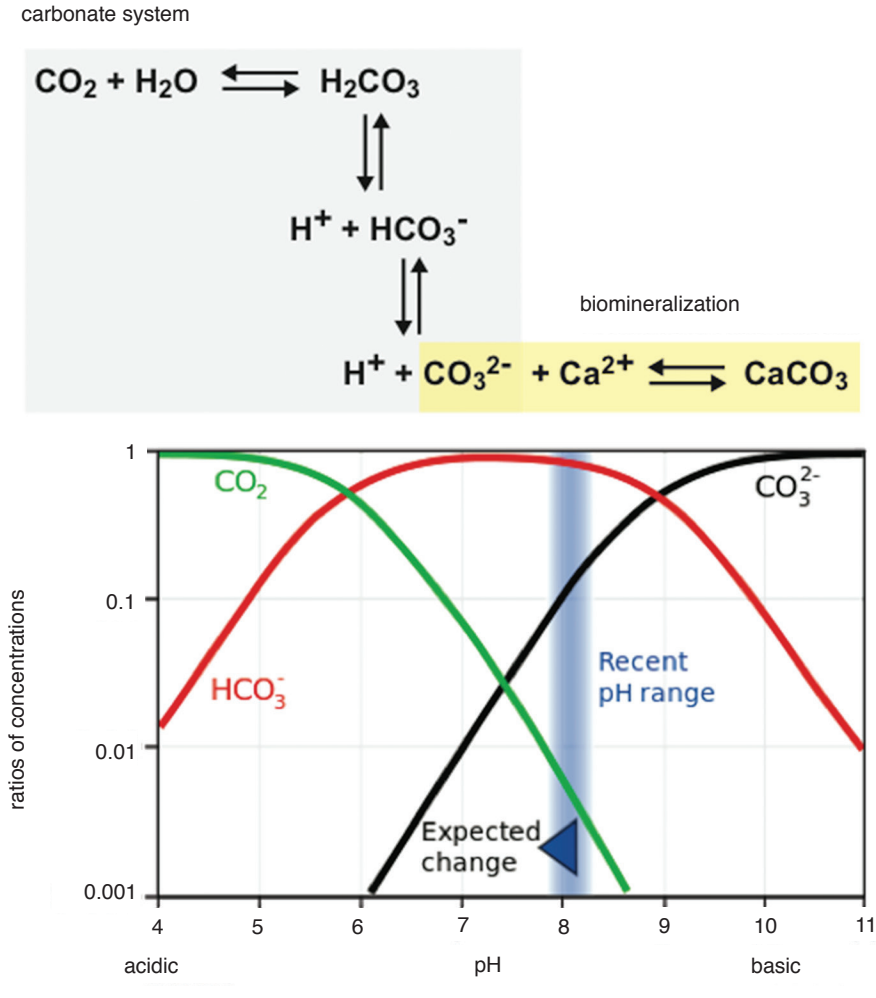

FIG. 3. The carbonate system in water and the effects of increased pCO_2 on acidity (pH) and on the balance of major constituents of the carbonate system (Wikimedia Commons).

state of the carbonate system: CO_2 (aq) + $H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+$. The carbonate system is the major source of CO_3^{2-} used for calcium carbonate synthesis, whereas metabolism contributes about 10% of the carbon found in shell carbonate, depending on species and age (MCCONNAUGHEY & GILLIKIN, 2008; BUTLER & others, 2011; BEIRNE, WANAMAKER Jr., & FEINDEL, 2012). Hence, the state of the carbonate system is of utmost significance for the ability of bivalves to grow and maintain calcareous shells, because it directly affects the calcium-carbonate saturation state of the water (see above).

The oceans have taken up about 40% of all anthropogenic CO_2 emissions so far (ZEEBE & others, 2008). The additional load of CO_2 is already affecting the ocean carbonate system, causing a decrease in ocean surface pH by about 0.1 units—i.e., ocean acidification and a corresponding reduction in carbonate-ion concentration (Fig. 3; ORR & others, 2005). This ocean acidification will continue for the foreseeable future, and its duration and severity

will depend on future CO_2 emissions (Zeebe & others, 2008). Model studies indicate that the carbonate saturation depth will continue to rise, and polar ocean surface waters may become undersaturated with respect to aragonite around 2050 (Orr & others, 2005). In fresh waters, acidification by anthropogenic CO_2 plays a minor role because their acidity is mainly determined by the surrounding geological and soil settings (Canfield & Hoyer, 1988). However, owing to the natural, long-term soil acidification and to such factors as acidic rain (caused by burning of fossil fuels) or reduction in atmospheric calcium input, the calcium ion concentration of many boreal oligotrophic lakes is slowly decreasing, together with the pH value of the water (Jeziorski & others, 2008).

The shift in chemical balance caused by elevated CO_2 concentrations (pCO_2) does affect biogenic calcification. Recent research has focused on pelagic calcifiers that play a significant role in the global carbon cycle. Most experiments demonstrated a negative effect of ocean acidification on calcification in pteropods (Orr & others, 2005), coccolithophorids (Riebesell & others, 2000), but enhanced calcification has also been reported in the coccolithophore *Emiliania huxleyi* (Lohmann, 1902) (Iglesias-Rodriguez & others, 2008).

The effects of ocean acidification on bivalve shells have been studied in only a few species. Several of these indicated severe and damaging effects on early larval development, particularly on larval shell development: for example, in the pacific oyster *Magallana gigas* (Thunberg, 1793), and the mussel *Mytilus galloprovincialis* (Lamarck, 1819 in 1818–1822) (Kurihara, Kato, & Ishimatsu, 2007; Kurihara & others, 2008). In adult mussels, *M. edulis*, biomineralization rates decreased and shell dissolution increased when exposed to elevated pCO_2 (Berge & others, 2006; Gazeau & others, 2007; Melzner & others, 2011), and the closely related species *M. galloprovincialis* suffered an overall decrease in metabolic activity and growth under long-term exposure to high pCO_2 corresponding to a pH of 7.3 (Michaelidis & others, 2005). Adults of the oyster *Crassostrea gigas* also showed a decrease in calcification rates with increasing pCO_2 (Gazeau & others, 2007). The ocean quahog *Arctica islandica,* however, seems to be unaffected by pCO_2 levels predicted by the International Panel on Climate Change models for the next 100 years. Stemmer, Nehrke, and Brey (2013) found no differences in growth rate or shell microstructure in animals exposed to elevated pCO_2 (up to 1120 µatm) for three months. Apparently, different species, and maybe even different populations, exhibit different levels of tolerance or preadaptation to ocean acidification. Furthermore, the impact of ocean acidification on shell formation may be modulated or amplified by reduced food supply (Melzner & others, 2011), by hypoxia (Melzner & others, 2013), or by other factors not presently considered. All in all, there is no consistent pattern in bivalve response to ocean acidification. Furthermore, ocean acidification events of the last 300 million years of earth history are not easily transferable for future projections of the current anthropogenic ocean acidification (Hönisch & others, 2012). Therefore, the extent to which the predicted changes in ocean chemistry and physics (warming) will affect bivalve populations on a global scale is uncertain (Gazeau & others, 2013).

BIVALVE METABOLISM: FEEDING, DIGESTION AND EXCRETION

Most modern bivalves in the subclass Autobranchia, infraclasses Pteriomorphia (mussels and oysters) and Heteroconchia (clams), feed on suspended or loosely deposited particles that are sucked into the mantle cavity by a water current generated by ciliary movements on the gills (ctenidia), whereas most modern bivalves in the subclass Protobranchia feed on deposited particles collected by the palp proboscides. In the former case,

the current generated by ctenidial cilia enters the mantle cavity directly through an incurrent aperture (oysters, scallops) or siphon (mussels and clams) and passes through the gills before being extruded through an excurrent aperture or siphon. The intensity of the water flow is determined by the degree of valve opening and the neuro-physiologically controlled intensity of ciliary movements. Neurotransmitters, such as 5-hydroxytryptamine, are released from the branchial nerve and activate ciliary beating through phosphorylation of cAMP (cyclic adenosine monophosphate)-dependent protein kinases (Murakami, 1987). Absorbed particles are either hydromechanically trapped or engulfed in mucus nets and sorted according to size and shape through the ciliary filter of the gills (or palps).

FOOD SELECTION

Filter-feeding bivalves ingest highly nutritional, protein-rich organic particles (such as bacteria, phytoplankton, and detritus) and smaller animal parts. The majority of the ingested particles have diameters of 4–50 µm, though some smaller (<1 µm) and larger particles (up to 200 µm)—consisting of free-living bacteria (0.2–2 µm) and silt particles coated with biofilms and refractory nitrogen-rich organic matter—are frequently mixed into the ingested fraction. The organic particles are retained and transported in a water-mucus slurry that flows along the digestive passageway over the gill filaments to the oral grove and esophagus into the gut (Jørgensen, 1996; Ward, 1996). Together with the food items, bivalves ingest inorganic particles, such as sand grains, that are mostly rejected as mucus pseudofeces before entering the digestive system. Sorting of trapped particles into edible and non-digestible items occurs on the palps and gills, primarily through rejection of pseudofeces. Rejected particles are entangled in high-viscosity mucus produced by mucocytes located in the frontal parts of the gill filaments (Beninger & Veniot, 1999; Ward & Shumway, 2004). The mucus strings or balls are transported over the gills onto the palps and from there dislodged to the environment.

Deposit-feeding protobranch bivalves, such as Nuculoidea and Nuculanoidea, transport sedimentary particles directly from the palp proboscides to the mouth, with sorting only by the palps. Certain other protobranch bivalves, such as the Solemyoidea, may largely or entirely replace deposit feeding with bacterial chemosymbioses. Similarly, bacterial symbioses may supplement or replace filter feeding in some lucinoid heteroconchs (Taylor & Glover, 2010).

FOOD SELECTION IN FILTER-FEEDING BIVALVES AT DIFFERENT SESTON CONCENTRATIONS

Food selection and rejection in terms of pseudofeces production increases when filter-feeding bivalves are exposed to high amounts of particles with low food quality. Since sorting and rejection of particles consumes 15%–20% of total energy expenditure, (Widdows & Hawkins, 1989), these processes can become a losing proposition for the bivalves when the concentrations of inorganic particles are too high or when concentrations of organic food particles alone are too low. Thus, valve closure and reduced filtration has been observed in several bivalves under very low (and, hence, more natural) algal concentrations, a behavioral adaptation that supports reduction of metabolic rate during times of low food supply (Riisgård, Kittner, & Seerup, 2003). Compared to infaunal bivalves, epifaunal bivalves seem to have special capacities for positively selecting organic food from the total seston at moderately high (<100 mg l^{-1}) concentrations (Hawkins & others, 1998). At extremely high seston loads, filtration becomes less selective, and the animals start ingesting high amounts of sedimentary material. Recently, an age-dependent response to elevated seston loads was observed in Antarctic infaunal, soft-shell clams, *Laternula elliptica,* exposed to

very high seston concentrations (Philipp, Husmann, & Abele, 2011). Large specimens were shown to close the siphons and reduce respiration rates when exposed to too-high inorganic particle loads near melting glaciers releasing plumes of fine-grained lithogenic material into the marine environment. Interestingly, smaller and younger animals that were still rapidly growing did not show this energy-saving behavior.

In the deep sea, many organisms have unusually long guts for food retention and nutrient extraction.This adaptation is seen in deep-sea, deposit-feeding bivalves, but not in deep-sea, filter-feeding bivalves. Instead, adaptations in filter-feeding bivalves are seen as enhanced gill surface area with increasing depth, thereby conferring extremely high clearance rates of scanty food to support bivalves with extremely low maintenance metabolic rates (Oliver, 1979; Järnegren & Altin, 2006).

DIGESTION

Food that enters the gut either as particles or engulfed in mucus strings is preprocessed in the stomach by mechanical cell rupture and extracellular digestion. Further sorting of particles occurs in the ciliated sorting grooves of the stomach, with particles of high nutritional value passed through the digestive gland via the digestive diverticula (Purchon, 1971) and those of little or no nutritional value (sediment grains) passed directly on to the intestine to be released as feces. The gelatinous crystal style, which rotates within the stomach against the gastric shield, releases the enzymes necessary for extracellular digestion. These enzymes are mainly carbohydrases such as amylases, necessary for the digestion of the mucus-food conveyor belt, but also include esterases involved in lipid-ester cleavage, alkaline and acidic phosphatase involved in phagocytosis, and low-activity proteases such as trypsin. The acidic pH (~5.6) frequently recorded in the stomach during low tides results from the release of acidic waste products from the highly acidic digestive gland into the stomach lumen and supports digestive activity (Langton, 1977; Morton, 1977). As the animals start filtering during tidal submersion, the gastric fluid becomes diluted with seawater, which increases pH to neutral values and stops the dissolution of the style and food digestion in the stomach. Apparently, in epibenthic, intertidal bivalves such as *Mytilus edulis,* digestive activity in the stomach is linked to the tidal cycle and, therefore, highest during low-tide emersion and valve closure. During this period extracellular food-processing results in the shortening to complete dissolution of the crystalline style, the main source for extracellular digestive enzyme activity in the bivalve gut (Langton, 1977). The style subsequently re-forms during the high-tide submersion phase (Morton, 1977).

Although there are common patterns in digestive enzyme activity distribution throughout different compartments of the bivalve gut—the crystalline style and stomach, digestive gland, and intestine—these vary between epibenthic and infaunal species, depending on the quality and nature of the food in each environment. Generally, carbohydrase (amylase, laminarase, cellulase-complex) activity dominates in the gut-style area, whereas carbohydrase and protease activity are relatively equal in digestive gland cells. Labarta and others (2002) showed that the infaunal bivalve *Mulinia edulis* (King, 1832 in King & Broderip, 1832) displays significantly higher protease activity in both organs than the epibenthic *Mytilus chilensis* (Hupé, 1854), in order to better exploit the lower protein content of the more refractory seston available in their subtidal habitat. Cellulase and laminarase activities were also much higher in *M. edulis* than in *M. chilensis* crystalline style, which is attributed to a higher proportion of macroalgal detritus in subtidal infauna diet (Labarta & others, 2002). The digestive enzyme profile changes when both species are transferred to a new habitat with different nutritional composition. Lysozymes that hydrolyse peptidoglycans of bacterial cell walls are secreted from

the crystal style into the gut lumen of epibenthic bivalves—*M. edulis, Modiolus modiolus* (Linnaeus, 1758 in 1758–1759) and *Chlamys opercularis* (Linnaeus, 1758 in 1758–1759)—and lysozyme-like activity is also measurable in digestive gland cells with highest values occurring in June and the lowest in February (McHenery, Birkbeck, & Allen, 1979; McHenery, Allen, & Birkbeck, 1986). As lysozyme activity is mostly concentrated in the digestive organs and to a minor extent is also present in the gills, it seems that its function is rather nutritional for the digestion of bacteria and, to a lesser extent, represents an antimicrobial defense mechanism.

Digestive enzyme activities in bivalves vary with environmental food availability and temperature and are, therefore, higher in summer than at the end of winter. Based on their investigations of food exploitation in blue mussels, Bayne, Hawkins, and Navarro (1988) modeled the adjustments of digestive efficiency through prolongation of gut-passage time to compensate for the slow filtering capacities of the mussels at the end of winter (March). Whereas ingestion rate in March was limited to only one-third of the summer (June) values, gut-passage time tripled. Nonetheless, the efficiency of food absorbance was lower in March than in June due to the reduced digestive enzyme activities in winter, resulting in lower net growth efficiency. Modifications of food ingestion, gut-passage time, and digestive intensity (enzyme activity) to compensate for reduced nutritional value of the seston, especially during the summer growth seasons, are possible, but rather as an adaptation to changes in feeding regimes on daily or weekly time scales (seasonal changes or storm events). Short-term enhancement of digestive efficiency for food-pulse processing could possibly be accomplished when digestive-gland enzymes are released to the stomach or intestine (Morton, 1983).

DIGESTIVE INVOLVEMENT OF GUT BACTERIA

The interactions and symbioses of bivalves with their gut-associated microflora have been discussed in several papers that highlighted the idea that the bacteria of the gut biofilm take over part of the host's digestive enzyme activity (for review, see Harris, 1993), especially with respect to cellulase activity. The term cellulase refers to a complex of cellulose-hydrolyzing enzymes—which includes cellobiohydrolases (E.C. 3.2.1.91), endoglucanases (E.C. 3.2.1.4), and -glucosidases (E.C. 3.2.1.21)—necessary to decompose lignocellulose biomass—i.e., wood (Honein & others, 2012). The wood-boring shipworms (Teredinidae) are specialized in digesting and feeding nearly exclusively on wood. Whereas early studies identified cellulose-digesting gut bacteria in the shipworm *Teredo navalis* (Linnaeus, 1758 in 1758–1759) as the main providers of lignin-degrading cellulase activity, later investigations removed microbial cellulase activity using antibiotics, indicating the enzymes were also endogenous to the bivalve gut and digestive gland cells (Payne, Thorpe, & Donaldson, 1972). Indeed, recently generated bivalve gene libraries found evidence for transcription of different cellulose-degrading enzymes in shipworm whole-body extracts, which align with sequences obtained from several other bivalves (Honein & others, 2012). The endogenous cellulase enzyme activity is not limited to wood-boring shipworms; Payne, Thorpe, and Donaldson (1972) detected endogenous β-glucosidase activity in the digestive gland, midgut, and even in the crystalline style (poly-β-glucosidase) of the deposit-feeding *Scrobicularia plana* (da Costa, 1778) (superfamily Tellinoidea). However, microbial symbionts do, indeed, play a major role in bivalve cellulose digestion: Harris (1993) provided a long list of gut microbe enzymes that support food digestion in aquatic invertebrates, including bivalves, crustaceans, and others. In the context of wood digestion, it is interesting that bacterial symbionts in the Deshayes gland, special to the shipworm gill lamella are involved in lignin breakdown, nitrogen fixation, and amino acid synthesis. This is

important to balance the unfavorably high C:N diet of several species of wood-feeding teredines (Waterbury, Calloway, & Turner, 1983). As bacteria are generally richer in nitrogen with lower C:N ratios than phytoplankton (3.5 compared to 6.6, cited from Gosling, 2003), and better meet the nitrogen requirements of the animals themselves, bacteria can constitute a considerable part of the bivalve diet, especially in eutrophicated estuaries and salt marshes.

PROTEIN UPTAKE, METABOLISM, AND COSTS OF GROWTH

On average, 50% of bivalve tissue dry mass is composed of protein (see Table 1), which carries the highest metabolic costs for synthesis, 9–13 J mg^{-1} protein. Proteins ingested with (algal) food have to be catabolized in order to serve as building units for bivalve tissue protein. Costs of protein synthesis and turnover, therefore, contribute significantly to metabolic rate, especially in younger animals in the logarithmic phase of somatic growth and in larvae. Fed the same algal diet, small, fast growing *Mytilus edulis* (10 mg dry body mass) achieve 10%–30% higher protein retention than fully grown mussels (Hawkins & Bayne, 1991). Net protein deposition in young mussels increases linearly with the rate of food ingestion, up to a state in which filter feeding, digestion, and protein synthesis consume too much of the animal's energy reserves (ATP) for the resulting increase of heat dissipation—i.e., specific dynamic action (Hawkins, Widdows, & Bayne, 1989). This explains why the scope for growth is sensitive to alterations in protein-synthesis efficiency (Bayne, 2004). In other words, bivalves that (are bred to) minimize the costs for protein synthesis and maintenance grow faster, a concept used to optimize commercial brood stocks. As almost always in biology, under conditions where something (in this instance, food) is plentiful, quality becomes less important. The beneficial effect of protein-enriched algal diets is limited to a level at which surplus ingested protein is exceedingly excreted in order to avoid a deleterious amino acid composition in bivalve tissues (for review, see Hawkins & Bayne, 1991). Thus, under conditions of plenty, total energy absorbance (caloric value), rather than protein content of the food, limits bivalve growth. Another question is how dietary proteins (amino acids) might improve the efficiency of protein synthesis in bivalves under energetically limited conditions. During starvation, the ultimate energy source is the decomposition of tissue protein, and bivalves usually suppress maintenance metabolism during times of foreseeably reduced food availability (winter), rather than metabolizing their tissue proteins. Bacteria-enriched detritus and carnivorous diets, especially in deep sea bivalves, may have positive effects in lowering the costs of protein synthesis because detrital material is ingested in a state of advanced decomposition.

Bivalves are generally herbivorous or detritivorous filter feeders that occasionally take up animal material. Some carnivorous bivalves, however, are specialized for flinging their incurrent siphon tip at smaller prey animals to suck in copepods and nematodes as preferred food. Surveys of stomach contents in abyssal bivalves revealed carnivorous alimentation in one-half of the specimens (Knudsen, 1970). A comparative study by Reid (1977) showed the same suite of extracellular digestive enzymes prevails in the stomach fluid of the carnivorous septibranch *Cardiomya planetica* (Dall, 1908) and detritivore- or phytoplankton-feeding bivalves, but with much higher activity in the protein-digesting enzyme fraction.

Dissolved organic matter, especially amino acids, are another easily metabolizable carbon source for bivalve protein synthesis. The most abundant amino acids in the dissolved carbon pool (serine, alanine, glycine/threonine, ornithine, and aspartic acid) constitute around 80% of the total dissolved amino acids taken up by *Mytilus edulis* in the North Sea (Siebers & Winkler, 1984). Veliger larvae of mussels and oysters were shown to absorb glycine (*Crassostrea gigas*) and alanine (*M. edulis*) from seawater

over membrane transporters at rates one order of magnitude higher than in adult bivalves (Manahan, 1983). Although the larvae can satisfy only about 10% of their energetic demand for growth through uptake of dissolved amino acids, this may represent one way by which they reduce costs for catabolism and enhance the efficiency of protein synthesis for their enormous growth requirement. The giant limoidean *Acesta excavata* (J. C. Fabricius, 1779) is a North Atlantic deep-sea species that has gills with a conspicuously large surface area. It colonizes continental margins and deep-sea cliffs and is often associated with deep-sea cold-water coral reefs of the scleractinian coral *Lophelia pertusa* (Linnaeus, 1758 in 1758–1759). Dissolved organic matter, including the nitrogen-rich mucus (carbon:nitrogen ratio of 5–7) released by the corals is rapidly dissolved and stimulates microbial growth in the vicinity of the reef (Wild & others, 2008). The close association of *A. excavata* with the reefs suggests that either the bacteria or the dissolved carbon fraction (or both) represent an additional food source for these deep-sea bivalves.

LIFETIME PATTERNS OF ENERGY STORAGE AND EXPENDITURE

Bivalves store energy in form of lipids and complex carbohydrates, such as glycogen, for gonad production and to satisfy enhanced needs of anaerobic activity, such as burst swimming in scallops and other mobile species. Furthermore, bivalves with enhanced tolerance of environmental hypoxia store high amounts of glycogen in the whole body, specifically in the adductor muscle (de Zwaan & Wijsman, 1976). In faster-growing species (or in individuals of a species bred for fast growth), storage of protein is clearly favored over lipids, and gonad maturation is delayed compared to slower-growing species and individuals that accumulate proportionally more lipids for earlier reproduction (for review, see Bayne, 2004). Therefore, protein metabolism is dominant in bivalves until the size of maturity is reached and the animals start to reproduce. Once maturity is reached, somatic body growth slows and, especially in long-lived species, eventually diminishes to negligible rates.

The metabolic rate in mature animals is the sum of tissue maintenance and activity (e.g., swimming, digging, ciliary beating) plus the costs for reproduction. Especially in such fast-growing and intensively spawning species as tropical scallops, trade-offs between tissue maintenance, mobility, and stress defense, on the one hand, and gonad maturation on the other hand, are impressive. As an example, exposure to predators delayed spawning in the catarina scallop *Argopecten ventricosus* (Guerra, Maeda-Martínez & others, 2012), as the animals allocated energy to the growth of the swimming muscle instead of gonad development. A major link between capacities for mobility and reproductive power is through nitrogen-rich guanidine derivatives, special amino acids derived from guanidine that can either form poly-guanidines, such as spermine and spermidine in support of reproduction, or build up the phosphagen pool in the muscle tissue that fuels burst activity (Gasparini & Audit, 2000).

Temperature, food availability, and stress factors (predators, infections, and toxicants) are the major environmental regulators of growth in bivalves (Gosling, 2003; Bayne, 2004). Maximal growth rates that can be attained in the species-specific thermal optimum range are constrained by limitations in food supply, which can, to some extent, be balanced by adjustments of food-assimilation rates and the efficiency of sorting the particles with the highest nutritive values (Fig. 4). Stress increases maintenance metabolism for cellular damage removal and repair.

HOW INTERTIDAL BIVALVES DEAL WITH OXYGEN DEPRIVATION AND OVER-OXYGENATION

Marine coastal environments can be low in oxygen, a situation typical of many estu-

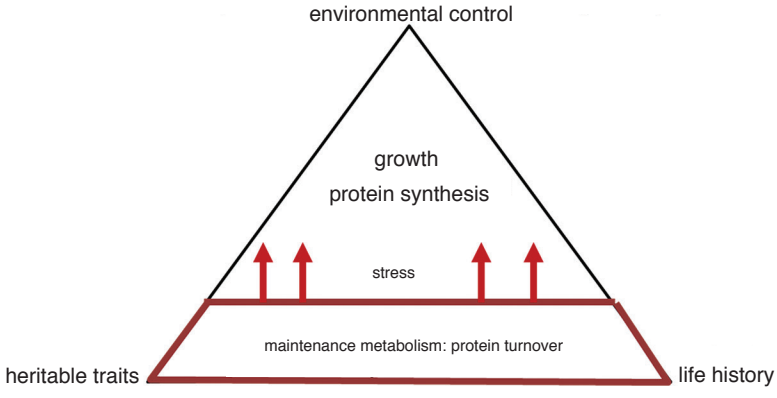

FIG. 4. Diagram showing interplay between environmental controls (temperature, food availabilty and composition, and stress factors), genetics (efficiency of protein synthesis, mobility type, gill size); and life history (life stage, age, reproductive state) (new).

aries, swamps, and tidal flats, which receive high amounts of organic matter from coastal runoff. Especially during stagnant phases, ventilation of surface sediments ceases so that hydrogen sulfide from microbial activity can diffuse upwards in the pore water and consume oxygen in the sediment surface and even in the sediment-water interface. This produces hypoxic or anoxic areas in which the geochemistry of the pore water is completely changed. Conditions worsen during warm summers when benthic biological and chemical oxygen demands increase. Increasing awareness of eutrophication and oxygen deficiency in European seas and aquatic ecosystems in the 1970's and 1980's prompted a large number of investigations into the response of marine macrofauna and demersal fish to oxygen deficiency (DE ZWAAN & others, 1991; WU, 2002; CLARKE & others, 2006). This resulted in a better understanding of the multiple strategies employed by marine and fresh water ectotherms for surviving periods of oxygen deficiency.

Indeed, oxygen is a double-edged sword for hypoxia-tolerant animals from watery or sedimentary habitats (ABELE, 2002; MASSABUAU & ABELE, 2012). Whereas oxygen is the most energetically efficient electron acceptor in mitochondrial respiration—and thus in aerobic-energy production—it is precisely the process of cellular respiration that causes the formation of reactive oxygen species (ROS), incompletely reduced and thus potentially harmful oxygen derivatives carrying odd numbers of redox-active electrons. During the evolution from exclusively anaerobic to aerobic life forms, organisms developed multiple mechanisms by which to exclude, bind, and generally control oxygen in cells and tissues, without letting tissue pO_2 drop below the line of oxygen deficiency (MASSABUAU, 2003; JAMES & SYED, 2012). Many of these strategies and mechanisms can be studied in members of the highly diverse molluscan clade.

Bivalves have open circulatory systems, so that their tissues are theoretically exposed directly to the vagaries of environmental oxygen concentration in marine and freshwater habitats. In reality, however, the bivalve shell enables its occupant to behaviorally adjust the internal environment with respect to dissolved gases, nutrients and the composition of bacterial communities in the vicinity of the soft tissues (ABELE & PHILIPP, 2012). Bivalves intermittently ventilate their fluid-filled mantle cavity through the siphons or by valve-opening movements. Mantle-cavity water and hemolymph contain oxygen and ions, metabolic products (CO_2 and other metabolites), and chemical-signaling molecules that exert concentration-dependent effects on the

animals' metabolic processes. In other words, each bivalve creates its own inner world that can differ dramatically from the outside chemical environment (either column or interstitial water), especially with respect to salinity (Davenport, 1979) and oxygen content (Abele & Philipp, 2012). Oxygen diffuses into the hemolymph (the blood equivalent of animals with open circulatory systems) via the gills and is directly taken up over the mantle surface into the tissue, following outside-to-inside pO_2 gradients (Massabuau & Abele, 2012).

The intensity of oxygen uptake is determined by the hemolymph perfusion of the gills (which, in turn, depends on cardiac output: heartbeat rate × filling amplitude) and by tissue consumption of oxygen (metabolic rate). As cardiac output is difficult to measure in bivalves because the heart is so small, most investigations rely on measuring heart rate in response to diminishing mantle-cavity water oxygenation. In slowly progressing mantle-water hypoxia in animals with open shells that experience progressive environmental hypoxia, as well as in rapid and linearly progressing hypoxia upon shell closure, a short period of increased heart rate (tachycardia) precedes slowing of heart rate (bradycardia). Bradycardia sets in at the low critical oxygen partial pressure, below which respiration is pO_2 dependent (oxyconforming). Clearly, the response of heart rate depends on changes in pO_2 instead of changes in pCO_2 in mantle-cavity water, which increases only during prolonged shell closure (Taylor, 1976b).

Numerous publications have demonstrated that bivalves from periodically hypoxic environments maintain respiratory independence against variable and diminishing ambient oxygen levels by inducing ventilation and circulation of mantle-cavity water in order to maintain a sufficiently oxygenated (perfused) state of their tissues (Bayne, 1971; Taylor & Brand, 1975; Brand & Morris, 1984; Strahl, Brey, & others, 2011). Coleman (1973) was one of the first to show that respiration rates and heart beat frequency in air-exposed *Mytilus edulis* are lowered compared to the immersed state. He also reported that metabolic rates of air-exposed mussels vary as a function of transient valve opening and air gaping. Thus mussels sense and, as much as possible, avoid complete and long lasting anoxia and transgression to an entirely anaerobic metabolism. Only when environmental pO_2 falls persistently below species-specific critical pO_2 values, do ventilation, circulation (i.e., cardiac output), and ciliary beat-frequency decline, and many bivalves enter into a state of metabolic rate depression (or suspended animation) that can be 10% or even less than the normoxic resting metabolic rate (see Storey & Storey, 1990). Metabolic-rate depression is characteristic of several groups of hypoxia-tolerant animals, including mollusks, shrimp, fish, hibernating vertebrates, and mammalian hibernators (see Storey & Storey, 1990, 2004a). It involves coordinated shutdown of glycolytic rates and energy-consuming processes, including (1) Na,K-ATPase activity and proton transport; (2) membrane-permeability arrest and cellular protein turnover, including protein synthesis; and (3) degradation and disintegration of ribosomal aggregates.

During environmentally induced depression of metabolic activity, hypoxia-tolerant bivalves reduce transiently dispensable energy-consuming processes and switch on more energy-efficient, anaerobic pathways in the mitochondria to achieve higher ATP gain per unit of glucose, avoiding a Pasteur effect (less energy-efficient, lactate-forming glycolysis involving rapid depletion of glycogen in anaerobically working muscle fibers) and conserving glycogen reserves in the tissues (Shick, Widdows, & Gnaiger, 1988). This energy-saving behavior supports tissue maintenance and stress-induced gene expression. Although suspended ciliary beating and bradycardia accompany the metabolic shutdown, they account only for a minor part of the energetic savings. Jørgensen (1974) showed

that energy expenditure for ciliary beating in *Mytilus edulis* accounted for less than 1% of routine metabolic rate. In contrast, valve closure upon air exposure or direct transfer to an anoxic or hypoxic medium in an experiment resulted in a rapid linear decline of mantle-cavity water pO_2 in bivalves, so that the animals often transit from the normoxic (normal atmospheric oxygen pressure in breathing gas or water, i.e., 21 kPa) state to complete tissue anoxia in less than one hour. The rate of shell-water oxygen depletion depends on the metabolic rate of the soft tissue and, hence, on feeding state, temperature, and stress levels experienced by the animals. Under declining tissue oxygenation, shutdown of energy-consuming systemic and cellular processes allows metabolic demand to match the rapidly declining O_2 and ATP availability in an attempt to minimize the resulting energetic deficit. Compensatory increase of circulation is marginal (or nonexistent) as the animals sense the absence of oxygen (see discussion below) and curtail energy-consuming behaviors, such as movements, ventilation, food digestion, and biochemical maintenance.

Much confusion has arisen from attempts to categorize invertebrates and especially bivalves as either oxyregulators or oxyconformers, based on different experimental setups, mostly performing ad-hoc transfer of animals to hypoxic or anoxic seawater in closed beakers. Taylor and Brand (1975, p. 187) argued that "these terms more correctly describe the extremes of the variable capacity of a species to maintain respiratory independence" against variable environmental pO_2. Indeed, within a single bivalve species, pO_2-dependent respiratory patterns can differ between small and large individuals. As shown for *Glycymeris glycymeris* (Linnaeus, 1758 in 1758–1759) by Brand and Morris (1984), small specimens with high-energy demand for growth qualify more as oxyregulators, whereas larger, older animals are better able to be idle as pO_2 falls and show a more oxyconforming response to diminishing oxygen.

ADJUSTMENT OF SHELL-WATER pO_2 AND OXYGEN SENSING

Attempts to relate oxygen consumption to changes of environmental pO_2 are biased by the ability of many bivalves to adjust the pO_2 in the mantle-cavity water around the soft tissues to much lower levels than the environmental oxygen levels (Abele & Philipp, 2012; Brand & Morris, 1984). Indeed, the ability of bivalves to behaviorally adjust mantle cavity water pO_2 has been largely under-investigated and underestimated. Thus, many researchers assume that air-exposed bivalves become strictly anoxic and, in some special cases, this may indeed be true. However, many intertidal bivalves reduce or even prevent anoxia induced by valve closure, in shell water and tissues, by air gaping (e.g., *Cerastoderma edule* Linnaeus, 1758 in 1758–1759) or by keeping the valves marginally open (e.g., *Mytilus edulis*), permitting slow or pulsed water circulation and oxygen uptake. As Moon and Pritchard (1970) demonstrated, mantle-cavity pO_2 in *Mytilus californianus* (Conrad, 1837), drops to zero when the valves were clamped shut, whereas air-exposed mussels with untethered valves maintained 30% to 40% of normoxic oxygenation in mantle-cavity water through frequent valve opening and air gaping. Likewise, hemolymph pO_2 in the mussel *Geukensia demissa* (Dillwyn, 1817) and the cockle *Cerastoderma edule* is maintained nearly at immersion (ventilating) levels by air gaping during tidal emersion. Moreover, in experiments measuring the metabolic heat dissipation, air-gaping intensity in both species related perfectly to the aerobic share of total energy expenditure (for review, see Shick, Widdows, & Gnaiger, 1988). This is a vital response for bivalves that, when experimentally forced into prolonged anoxia (sudden transfer and more than twelve hours of exposure to oxygen-depleted seawater), mount a pronounced Pasteur effect (accelerated glycolysis and accumulation of acidic endproducts

of anaerobiosis; see GÄDE, 1975), an atypical response for cockles. Another anoxia-avoidance strategy is positioning on low shore levels and in moist and shaded micro-niches, to reduce valve-closure times during air exposure. In actively avoiding anoxia, bivalves may keep up growth and reproduction in intertidal areas and improve species-specific habitat exploitation through ecophysiological adaptation.

The same strategies are also employed by subtidal bivalves. Using implanted oxygen optodes, ABELE and others (2010) recorded shell-water pO_2 in marine mollusks: two mud clams, two scallops, and the limpet *Patella vulgata* (LINNAEUS, 1758 in 1758–1759) that were immersed in fully normoxic seawater and found it to be considerably lower than the pO_2 of the normoxic incubation water. As the range of mean shell-water pO_2 in each species corresponds to its routine metabolic rate (higher in scallops, lower in mud clams), ABELE and others (2010) hypothesized that bivalves adjust their shell-water pO_2 to a sufficiently low level to support their metabolic requirements and, at the same time, prevent over-oxygenation of their tissues. It even seems possible that some bivalves, such as the long-living ocean quahog *Arctica islandica*, self-induce metabolic rate depression by periodically burrowing into the sediment and, in so doing, reduce shell-water pO_2 to trigger metabolic slowdown (TAYLOR, 1976a; STRAHL, BREY, & others, 2011). Such behavior would require the existence of oxygen-sensitive chemoreceptors on the bivalve outer surface to sense shell-water pO_2.

Indeed, oxygen chemo-sensing in bivalves has been found to function through peripheral oxygen chemo-sensors, the osphradial neurons. In the tellinoidean *Donax* LINNAEUS, 1758 in 1758–1759, ciliated sensory structures (osphradia) are located at the tip and inside the inhalant syphon, as well as on the mantle edge, and have been ascribed chemosensory, rather than mechanosensory, functions (HODGSON & FIELDEN, 1984). Recently, the osphradial peripheral oxygen chemo-sensors that respond to hypoxia by dose-dependent increases in burst activity have been detected in the pond snail *Lymnaea stagnalis* (LINNAEUS, 1758 in 1758–1759) (JAMES & SYED, 2012). These peripheral oxygen chemo-sensors drive the activity of the effector neurons, the central pattern generators of heart beat in mollusks. Synaptic connections between peripheral oxygen chemo-sensors and effector neurons show plasticity in response to individual hypoxic exposure. This means that hypoxic adaptation will centrally alter the basal neuronal drive of the molluscan heart and, in so doing, set the baseline for aerobic metabolic rate in accordance with species niche-specific oxygen demand (high in scallops, low in mud clams).

The deep-burrowing, intertidal, soft-shell clam *Mya arenaria* (LINNAEUS, 1758 in 1758–1759) illustrates the ability to sense and respond to oxygen shortage. *M. arenaria* shows low metabolic rates (compared to more active clams, such as scallops and blue mussels) and survives periodic siphon closure during emersion without major energy deficits (RIISGARD, KITTNER, & SEERUP, 2003). Interestingly, this species maintains extremely low mantle-cavity pO_2, with a median value round 0.4 kPa, even when maintained in normoxic seawater; this contrasts with the median shell-water pO_2 values of *Arctica islandica* and *Pecten maximus,* 3.6 kPa and 8.3 kPa, respectively (ABELE & others, 2010). When buried under algal mats (and thereby deprived of their access to oxygen-enriched surface waters), *M. arenaria* burrows upwards and, by moving towards the sediment surface, widens the syphon diameter for enhanced shell-water ventilation to prevent complete and prolonged anoxic exposure (AUFFREY, ROBINSON, & BARBEAU, 2004).

Thus, bivalves sense the environmental oxygen levels and regulate ventilation and circulation to establish a favorable oxygenation around their tissues also under extreme conditions: too-high and too-low water oxygenation levels or air exposure. As observed by WIDDOWS and

Shick (1985), most experimental studies exposing intertidal bivalves to less than 12 hours of complete anoxia deliver valuable insight into physiological and biochemical response to prolonged anoxia, but do not represent ecologically realistic scenarios of aerial exposure during tidal cycles (maximal tidal emersion time is usually 6 hours).

HYPOXIA, ANOXIA, AND SULFIDE TOLERANCE

Marine and freshwater bivalves are among the most hypoxia-tolerant aquatic macrofauna (de Zwaan & Eertman, 1996), and some benthic infaunal bivalves survive days and even weeks in complete anoxia (Theede & others, 1969). Some studies even suggest increased sensitivity of mud clams to normoxic conditions which, for these animals, may appear hyperoxic. Joyner-Matos and others (2007) investigated population patterns of the freshwater clam *Sphaerium* sp. over its entire distribution range, from a hypoxic-acidic swamp system through the ecotone transition zone into an oxic-neutral pH stream site. These small (<9 mm shell length) clams proved highly tolerant of and adapted to the extremely hypoxic conditions in the swamp. Not only were population densities much higher in the swamp and the ecotone (intermediate) than at the stream sampling sites, but the ovoviviparous clams also had the highest reproductive output under the extreme hypoxic swamp conditions (Joyner-Matos & others, 2011). Fitness parameters such as RNA:DNA ratios were highest in the swamp area, and nucleic acid oxidation (a cellular stress indicator) increased between the swamp interior towards the margins and to the stream sites. Stress-induced gene expression along the sampling transect was highest in the swamp margins and the ecotone transition between hypoxic and oxic and lowest in the interior swamp control sites (where the animals were best adapted and least under stress). On average, clam size was larger and fecundity lower in the marginal population colonizing the edge of tolerance in the stream. These clams were suffering oxidative damage but were not able to significantly mount stress-induced gene expression of antioxidants and heat-shock proteins (Joyner-Matos, Downs, & Julian, 2006). This study demonstrates that hypoxia can be beneficial for certain bivalve species. The authors suggested that, especially in hypoxia-tolerant species with smaller body mass, the absence of cellular stress may translate into enhanced fitness at the organism level.

Several, larger burrowing mud clams are among the hypoxic champions, characterized by comparatively low standard and routine metabolic rates and an oxyconforming response to hypoxia with pronounced metabolic rate depression below the species specific p_c. Many of these species also maintain some level of anaerobic metabolism under normoxic conditions, as in *Arctica islandica* (see Brand & Morris, 1984) and the estuarine clam *Rangia cuneata* (G. B. Sowerby, 1832 in 1821–1834)(see Chen & Awapara, 1969). Some hypoxia-tolerant marine bivalves store oxygen in the hemolymph by binding it to cellular hemocyanin or hemoglobin or to huge and complex, freely floating hemoglobin molecules as an internal oxygen storage buffer (Weber, 1980; Kluytmans & others, 1983; de Zwaan & others, 1991).

All heterotrophic organisms maintained cellular capacities for anaerobic energy production throughout their aerobic evolution. Burst activity in vertebrate skeletal muscles is fueled through anaerobic glycolysis that ferments blood glucose (or glycogen) to pyruvate, which is then reduced to lactate by the enzyme lactate dehydrogenase (LDH). This kind of burst activity is costly, as it involves accelerated blood flow and high glycolytic throughput (Pasteur effect) that draws heavily on energy reserves and causes muscle fatigue through acidification from lactate accumulation (Ebberink & de Zwaan, 1980). Clearly, for facultative anaerobes this strategy is not an option for surviving prolonged scarcity

TABLE 2. Anoxic survival time of marine bivalves in LT_{50} experiments and known presence or absence of bacterial endosymbionts. Experiments by KLUYTMANS and others (1983) were terminated after 96 hours (four days) and thus do not represent LT_{50} values; *asterisk* (*), end of experiment with 100% survival; *double asterisk* (**), no survival after 48 hours of anoxic exposure; LT_{50}, experimental time span at which 50% of exposed animals have died (new).

Species	Anoxia LT_{50}	Metabolic rate depression	Endosymbionts	Data source
Arctica islandica (LINNAEUS, 1767 in 1766–1767)	55 days	1%	no	Theede & others, 1969
Astarte borealis (SCHUMACHER, 1817)	85 days	no data	no	Dries & Theede, 1974
Lucinoma aequizonata (SCHUMACHER, 1817)	262 days	4%	yes	Arndt-Sullivan, Lechaire, & Felbeck, 2008
Glycymeris pilosa (LINNAEUS, 1767 in 1766–1767)	>4 days*	2%	no	Kluytmans & others, 1983
Tellina planata (LINNAEUS, 1758 in 1758–1759)	<2 days**	20%	no	Kluytmans & others, 1983
Venus gallina (LINNAEUS, 1758 in 1758–1759)	<2 days**	6.4%	no	Kluytmans & others, 1983

or absence of oxygen—i.e., environmental hypoxia or long-lasting foraging dives. Hypoxia-tolerant bivalves usually show no pronounced glycogen-fueled effect, and they produce only very minor amounts of lactate (if any), even when facing severely hypoxic conditions (DE ZWAAN & WIJSMAN, 1976; BRINKHOFF, STÖCKMANN, & GRIESHABER, 1983; GRIESHABER & others, 1994; STRAHL, DRINGEN, & others, 2011).

When facing ambient oxygen deficiency (or other forms of stress), the primary strategy of hypoxia-tolerant bivalves, unlike vertebrates, is to reduce metabolic rate to between 5% and 10% of their aerobic resting metabolic rate by depressing both the consumption and the formation of metabolic energy (ATP). The extent to which metabolism can be reduced depends on species-specific lifestyles and on energetic requirements in the resting metabolic state. Slow-growing sessile clams have the highest capacities for metabolic rate depression, which correlates positively with their outstanding hypoxia and anoxia endurance. The marine clams *Arctica islandica* and *Glycymeris pilosa* (LINNAEUS, 1767 in 1766–1767), which reduce metabolism to 2% (or less) of resting levels, are good examples (KLUYTMANS & others, 1983; OESCHGER, 1990; Table 2).

Lucinoma aequizonata (STEARNS, 1890) owes its extreme anoxia endurance (see Table 2, LT_{50} values indicating the time span at which 50% of exposed animals had died) to the presence of sulfide-oxidizing bacterial endosymbionts in its gill tissues. These bacteria not only protect the animals from the toxicity of hydrogen sulfide (H_2S), a potent inhibitor of mitochondrial cytochrome c oxidase, but also supply huge amounts of chemoautotrophically produced glycogen as an energy source for the survival of their bivalve hosts in anoxic conditions. This glycogen is stored in the mantle tissue and enables the bivalve to actively burrow in anoxic and sulfidic mudflats (ARNDT-SULLIVAN, LECHAIRE, & FELBECK, 2008). Furthermore, in the strictly anoxic experimental setup, in which the final H_2S levels were up to 1 mM in the overlying seawater, gill tissue was strongly depleted as the animals digested their bacterial endosymbionts as an emergency food reserve.

Despite the foregoing example, hydrogen sulfide is generally poisonous to marine invertebrates, including bivalves without endosymbionts. However, tolerance to low-oxygen conditions and to hydrogen sulfide exposure is also adaptive in evolutionarily early organisms accustomed to dealing with

low-oxygen, sulfidic environments. In most marine invertebrates, including bivalves without sulfide-oxidizing endosymbionts, additional sulfide stress reduces hypoxic survival on average by 30% (Vaquer-Sunyer & Duarte, 2010). Hydrogen sulfide binds to heme-containing enzymes and respiratory proteins such as cytochrome c oxidase and hemoglobin. In-vitro inhibition of cytochrome c oxidase usually occurs at nanomolar concentrations, and 50% inhibition of the enzyme in vitro (K_i) is often reached at concentrations well below 0.5 µM sulfide (Jahn & Theede, 1997). The respiration of isolated gill mitochondria starts to be inhibited at higher concentrations (>20 µM sulfide), which indicates that the mitochondria have the capacity to detoxify H_2S at low concentrations, thereby shielding their respiratory-chain components from the toxic effects (Powell & Somero, 1986). Sulfide exposure not only compromises energy acquisition but leads to tissue acidification; sulfide also interacts with sulfhydril (SH) groups on the cysteine residues of proteins.

Bivalves defend against hydrogen sulfide in three major ways, which can be summarized as (1) exclusion, (2) oxidative detoxification in the mitochondria, and (3) endurance of prolonged exposure through metabolic depression and anaerobic energy production (Oeschger & Pedersen, 1994). Mechanical or chemical exclusion of sulfide in hemolymph and tissues is achieved by keeping shells tightly closed, with only occasional ventilation, and by precipitating sulfide on the outside of the shell or in the periphery of the tissues by reaction with transition metals such as iron. Bivalves collected in sulfidic sediments often have a black periostracum that lightens during recovery in sulfide-free water. Optically dense sulfide precipitation bodies contain hemeproteins or ferritin that bind and chemically oxidize sulfide in the periphery of the tissues. Sulfide-binding proteins, as well as the redox molecules (and acknowledged antioxidants) glutathione and ascorbate (vitamin C) can bind sulfide through formation of disulfide bridges (S=S) and function as a protective barrier against its toxic effects. Because adaptation to sulfidic conditions increases the concentration of both redox buffer molecules in the gills of the mussel *Geukensia demissa,* Doeller, Grieshaber, and Kraus (2001) proposed that these molecules act as a sink for electrons from sulfide oxidation in the absence of oxygen. In the vesicomyid, hydrothermal vent clam *Calyptogena magnifica* Boss & Turner, 1980, which hosts sulfide oxidizing endosymbionts in its gills, sulfide is taken up over the foot into the hemolymph, where it is bound to a nonheme serum protein that functions as a transporter molecule for safe delivery of toxic sulfide to the endosymbionts (Arp, Childress, & Fisher Jr., 1984).

The main sulfide-protection mechanism involves the oxidation of sulfide in the mitochondria by a sulfide oxidase, in which 2 moles of the electron-donor hydrogen sulfide (H_2S) are converted to the nontoxic and water-soluble thiosulphate ($S_2O_3^{2-}$) under aerobic (and even hypoxic) conditions. The nature of this sulfide oxidase is unclear, but it is likely to be a small hemeprotein, similar to the one found in the hemolymph of *Calyptogena* Dall, 1891. As illustrated in Figure 5, the sulfide oxidase feeds electrons into the mitochondrial electron transport system by reducing cytochrome c at complex III. Sulfide oxidation thus shortcuts the first two phosphorylation sites (complex I and II) of the electron-transfer system and produces 1 mole ATP per mole oxidized sulfide and oxygen consumed—i.e., P:O ratio of 1 (O'Brien & Vetter, 1990; Doeller & others, 1999). Doeller and others (1999) demonstrated that the ATP gained from sulfide oxidation contributes significantly to cellular work, such as the ciliary beating of the gills of the sulfide-adapted salt marsh mussel *Geukensia demissa*, indicating that cellular functions in bivalves are partly supported by hydrogen sulfide in the absence or in addition to other respiratory substrates. Oxygen is essential for sulfide oxidation and both high-sulfide concentrations and anoxic conditions abrogate mitochondrial electron

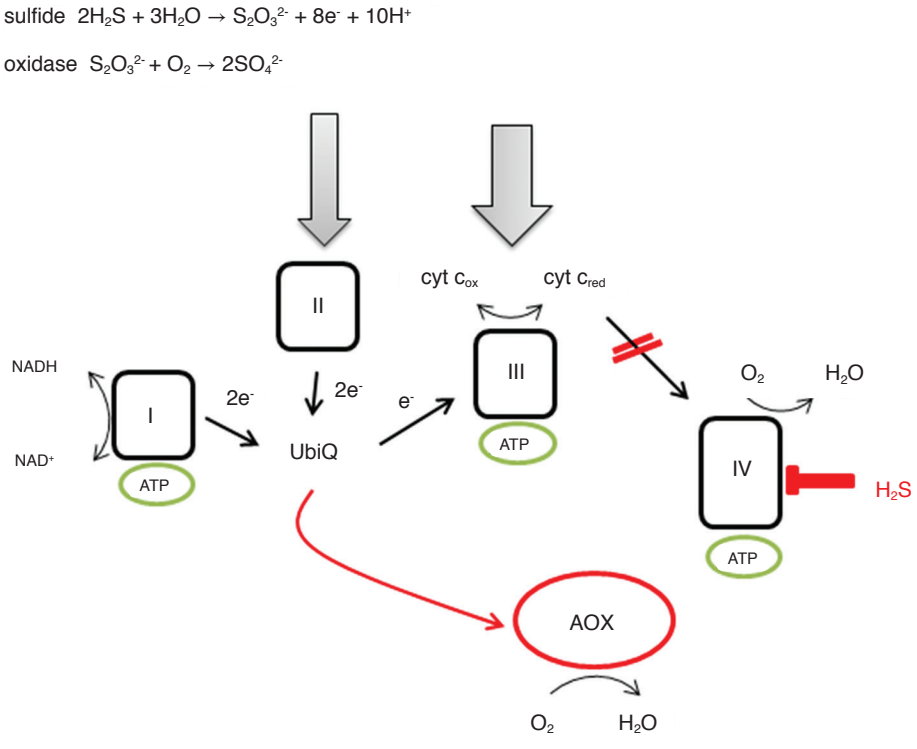

FIG. 5. Diagram of the mitochondrial electron-transport system (ETS) for sulfide oxidation. Electrons from sulfide are delivered either to cytochrome c (*cyt c*) at complex III or to ubiquinone (*UbiQ*) (as in the polychaete *Arenicola marina* (LINNAEUS, 1758 in 1758–1759)(see GRIESHABER & VÖLKEL, 1998). The alternative oxidase (*AOX*) receives electrons either directly from UbiQ or from cyt c. Depending on the location (complex II or III) at which the electrons enter the ETS and whether regular or alternative electron transport occurs, between 0 and <2 mol ATP can be generated per mol H_2S. The red pathway indicates cytochrome c oxidase ($cyt\ c_{ox}$) inhibition by H_2S that shunts electrons to the AOX, which is neither sulfide nor cyanide sensitive. *NADH,* nicotinamide adenine dinucleotide reduced; *NAD+,* oxidized form of nicotinamide adenine dinucleotide (new).

flow, leaving the respiratory-chain intermediates in a highly reduced state. This, in turn, presents a challenge during reoxygenation as the reduced intermediates, especially ubiquinone and cytochrome c, autoxidize to form incompletely reduced reactive oxygen species (ROS). MORRILL and others (1988) were the first to observe that thiobiotic marine meiofauna contain more antioxidant enzyme activity than their oxybiotic counterparts. Antioxidants such as glutathione can apparently have dual function as a primary storage buffer for sulfide-derived electrons and, in a later step, neutralize oxygen radical electrons in enzyme-catalyzed or spontaneous oxidation reactions.

Sulfide oxidation can take place in both the bivalve gill mitochondria and in their chemolitoautotrophic endosymbionts, as reported for the sulfide-tolerant protobranch *Solemya reidi* (BERNARD, 1980). *S. reidi* is specialized for burrowing in the highly reduced sediments of sewage dump sites at Santa Monica Bay (California, USA), where hydrogen-sulfide concentrations in pore water reach up to 3 mM (KRAUS, DOELLER, & WITTENBERG, 1992). Bacterial endosymbionts in the gills of *S. reidi* use either hydrogen sulfide or thiosulfate as reducing equivalents for ATP production and carbon fixation. In the absence of a sulfide-binding factor in *S. reidi* hemolymph, sulfide and

oxygen are taken up directly over the body surfaces (foot, mantle, gills). The lower section of the gill filaments is densely packed with bacteriocytes (Powell & Somero, 1985), where the electrons from hydrogen sulfide are directly transferred to reduce a special cytochrome c (cyt_{552}) of the bacterial electron-transfer system (Kraus, Doeller, & Wittenberg, 1992). Interestingly, Kraus, Doeller, and Wittenberg (1992) could not detect formation of a ferric-hemoglobin sulfide complex in the gill cells of *S. reidi*. In ferric (Fe^{3+})-hemoglobin, sulfide is reversibly ligated to the heme group, which enables the less sulfide- and anoxia-adapted congener *Solemya velum* (Say, 1822) to bind, store, and control free hydrogen sulfide in its tissues. Instead, hemolymph and tissues of *S. reidi* accumulate huge amounts of thiosulfate as an alternative, or additional, substrate for the bacterial electron-transfer system. O'Brien and Vetter (1990) observed the electron-transfer system of sulfide-oxidizing mitochondria of *S. reidi* tissues to be loosely coupled, which they interpreted as an advantage for sulfide oxidation/detoxification to proceed in the hypometabolic state in which ATP turnover/ADP availability is low. Electrons from sulfide or thiosulfate oxidation enter the mitochondrial electron-transfer system at the level of cytochrome c (complex III) or possibly ubiquinone (complex II), theoretically generating between 1 and 2 mol ATP per mol H_2S (or thiosulfate, Fig. 5), while the real rates are much lower (Doeller, Grieshaber, & Kraus, 2001).

As discussed above, sulfide oxidation in bivalves is both a mechanism of energy production and of sulfide detoxification. The low experimental ATP:oxygen ratio indicates that detoxification may be the function of primary importance. Apart from its inhibitory effect on cytochrome c oxidase, sulfide has been shown to be a potent uncoupler of the mitochondrial electron-transfer system because it abrogates the control of phosphorylation over the electron transport and accelerates sulfide oxidation in the absence of ADP (during hypometabolism). As long as the cellular H_2S concentration can be controlled below the critical concentration for cytochrome c oxidase function, ATP can be produced at the respiratory complex IV (Fig. 5, black arrows). Once cytochrome c oxidase becomes inhibited by sulfide, the electrons are shunted to an alternative oxidase (AOX) (Fig. 5, red arrow) that permits respiration to continue at a reduced pace. AOX was first discovered in plants and protists and is inhibited by salicylhydroxamic acid (SHAM) but not by cyanide, H_2S, or nitric oxide (NO), all of which inhibit the classical cytochrome c oxidase respiration pathway. Using SHAM, an AOX was confirmed in sulfide-tolerant annelid worms and bivalves, including *Arctica islandica, Geukensia demissa*, and members of other invertebrate phyla and subphyla (van Hellemond & others, 2003; McDonald, Vanlerberghe, & Staples, 2009). A taxonomic analysis by McDonald, Vanlerberghe, and Staples (2009) identified AOX sequences in many species from various phyla and suggested that AOX and a branched mitochondrial electron-transfer system represented an ancestral trait that has been lost in strictly aerobic vertebrate and arthropod genomes. Aside from its function as an alternative electron-transfer system pathway under highly sulfidic conditions, the AOX may facilitate the adjustment of the appropriate oxygen partial pressure in animal tissues (Massabuau & Abele, 2012). Due to its lower affinity for oxygen than cytochrome c oxidase, the AOX also continues to consume oxygen at higher pO_2. The AOX receives electrons from UbiQ, bypassing phosphorylation sites at complexes III and IV and generating either 1 ATP or none, depending on the entrance of electrons to the electron-transfer system: nicotinamide adenine dinucleotide (NADH) at complex I, succinate or H_2S at complex II. This pathway is thus relatively independent from the supply of ADP and thereby from muscular activity in the metabolically depressed state.

Whereas sulfide oxidation seems to be ubiquitous across bivalve species, detoxifica-

tion capacity increases through adaptation: thus, less-adapted species become poisoned at much lower concentrations of sulfide (Doeller & others, 1999). The importance of preadaptation was clearly evident in a comparison of different populations of the clam *Macoma balhtica* (Linnaeus, 1758 in 1758–1759), some from Baltic sulfide-rich habitats and others from North Sea habitats with low sulfide (Jahn & Theede, 1997). Whereas cytochrome c oxidase K_i values (nM sulfide concentrations causing 50% inhibition in vitro) hardly differed between the two populations, the sulfide-tolerant populations were better able to exclude and oxidize H_2S under aerobic conditions. These differences vanished when oxygen was absent during experimental sulfide exposure, so the authors concluded that sulfidic adaptation consisted of improved sulfide-exclusion and oxidative-detoxification mechanisms. Thiosulfate is nontoxic and water soluble and can accumulate in high amounts in tissues of sulfide-oxidizing species because diffusion to the environment is slow (reviewed by Völkel & Grieshaber, 1996). Alternatively, thiosulfate can be oxidized to sulfate by sulfur-oxidizing bacterial endosymbionts, as in *Solemya reidi* (see Kraus, Doeller, & Wittenberg, 1992).

METABOLIC REGULATION AND HYPOXIC GENE TRANSCRIPTION

The coordinated depression of metabolic rate in hypoxia-tolerant bivalves involves the down-regulation of glycolytic and respiratory ATP production and parallel suppression of a plethora of ATP-consuming cellular processes, including protein synthesis and energy-dependent membrane transport (Boutilier & St-Pierre, 2000; Storey & Storey, 2004b). Adenylate concentrations (mainly of ATP) become reduced during transition to the hypometabolic state, but with a relative preservation of the cellular adenylate energy charge (Wijsman, 1976). The adenylate energy charge is defined as the ratio of ATP equivalents over the sum of total adenylates in the cell: [ATP] + ½ [ADP]/[ATP] + [ADP] + [AMP] (for further reading, see Plaxton, 2004). This relative stabilization of adenylate energy charge keeps up the cellular membrane gradients and compartmentalization of metabolites, essential for cellular functioning in the hypometabolic state and is of special significance in bivalve gills where active (ATP-consuming) membrane transport is intense (Clark & others, 2013). The adenylate equilibrium is maintained by the adenylate kinase reaction, which regenerates ATP from ADP (2 ADP → ATP + AMP) and through transphosphorylation of phosphagen-derived monophosphate (molluscan phospagen).

Metabolic rate depression is rapidly and efficiently achieved by reversible protein phosphorylation of such glycolytic pacemaker enzymes as glycogen phosphorylase (GP), phosphofructo-kinase, pyruvate kinase (PK), and pyruvate dehydrogenase (summarized in Storey & Storey, 1990). A second mechanism is the allosteric modification of key enzymes as substrate levels change. PK was the first enzyme shown to be modified by reversible phosphorylation under anoxic conditions, and this mechanism has since then been documented in many bivalves and other facultative anaerobes. Under aerobic conditions, the dephosphorylated PK dominates and enables maximal glycolytic rates. The dephosphorylated aerobic PK shunts phosphoenolpyruvate (PEP) towards the citric acid cycle for aerobic energy production. Anoxia-induced phosphorylation of PK reduces enzyme affinity for PEP so that glycolytic flux is shifted towards phosphoenolpyruvate carboxykinase (PEPCK) to feed into mitochondrial anaerobic pathways that are typical for hypoxia-tolerant bivalves (see below). Anoxia-induced phosphorylation alters several other kinetic properties of PK, increasing the inhibitory effect of alanine, the anaerobic end product, and decreasing the sensitivity for allosteric activation by the glycolytic coactivator fructose-1,6-bisphosphate (F-1,6-P_2) (Storey & Storey, 1990).

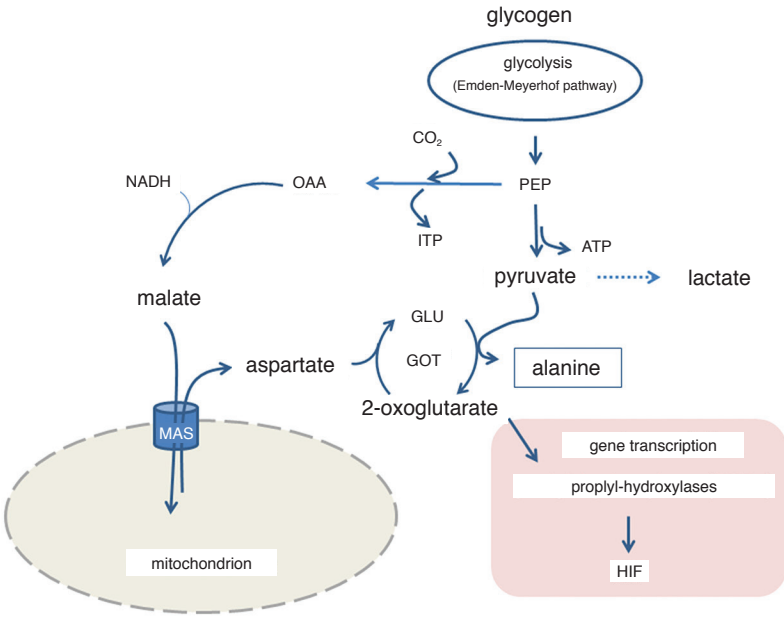

Fig. 6. Simplified metabolic chart of anaerobic energy pathways in hypoxia-tolerant bivalves; *GOT,* glutamic-oxalacetic transaminase; *GLU,* glutamate; *HIF,* hypoxia-inducible transcription factor; *ITP,* inosine triphosphate; *MAS,* malate-aspartate shuttle; NADH, nicotinamide adenine dinucleotide; *OAA,* oxaloacetate; *PEP,* phosphoenol-pyruvate (new).

There are numerous examples of reversible phosphorylation in anoxically exposed bivalves. Oeschger and Storey (1993) demonstrated a decrease of GP and PK activity and a corresponding change of PK-inhibition constants for alanine, ADP, and F-1,6-P_2 in *Arctica islandica* following ten days of anoxic exposure. They further tested the effect of anoxic hydrogen sulfide stress and found that sulfide exposure had no independent or additional effect on either protein phosphorylation or mitochondrial anaerobic-energy production. Likewise, in a survey of activity and reversible phosphorylation of glycolytic and pentose phosphate shunt enzymes in various tissues (gill, mantle, hepatopancreas, and adductor muscle) of anoxia-exposed oysters—*Crassostrea virginica* (Gmelin, 1791), exposed to 96 hours of experimental anoxia—Greenway and Storey (1999) revealed rapid, strong depression of PK maximal activity in all tissues, presumably through reversible phosphorylation. PEPCK was rather down regulated, except for mantle tissue where a 30% PEPCK activity induction occurred. A strong decrease of GP activity, also caused by reversible phosphorylation, was observed, indicating suppression of glycogen mobilization in the hypometabolic state. The oyster study covered summer and winter seasons (with all enzymatic determinations conducted at 21°C) and showed more intensive and widespread anoxic down-regulation of enzyme activities in winter, when metabolic rates were lowered by colder temperatures. Alterations at the levels of gene transcription were not investigated in this early study, but Greenway and Storey suggested that transcriptional adjustments take place during later phases of adaptation if the anoxic conditions prevailed.

While the case still has to be made for anoxia, at least in hypoxic conditions GP mRNA was up-regulated after twenty-four days of exposure in *C. gigas* (David & others, 2005). Interestingly, during the first seven days of hypoxic incubation, GP mRNA was

down-regulated in the gills, mantle, and digestive gland of *C. gigas,* which points to diminished glycogen mobilization in the early phase of oxygen shortage, during which the enzyme proteins are inactivated by reversible phosphorylation. Overall, the quantitative polymerase chain reaction (qPCR) study of David and others (2005) demonstrates a down-regulation of gene transcription at the onset of hypoxia (from zero to seven days), thus matching the concept of rapid onset of metabolic depression, whereas up-regulation of enzyme transcripts started only after two weeks of exposure to the hypoxic conditions. Delta-9-desaturase, metallothionein-like protein, and glutathione peroxidase (GPx) were among the up-regulated mRNAs at this later stage of the hypoxic experiment, all of them functionally important in hypoxia.

Many of the seminal papers investigating the glycolytic and mitochondrial metabolic response to anoxic exposure (environmental anaerobiosis) in bivalves have used the blue mussel *Mytilus edulis* or species of the oyster *Crassostrea* (Sacco, 1897). Both are easily obtained and have considerable tolerance to both hypoxia and anoxia, as they are periodically exposed to air during low tides. Blue mussels store between 5% and 20% of glycogen adductor muscle biomass as an energy resource that supports for anaerobic glycolysis. Instead of lactate, alanine is the major anaerobic metabolite accumulating in bivalve adductor muscle during the onset of environmental anaerobiosis (Ebberink, Zurburg, & Zandee, 1979; Zurburg & Kluytmans, 1980). Marine bivalves use aspartate from the muscular pool of free amino acids as NH_2-donor for the transamination of glycolytic pyruvate, resulting in an increase of muscle alanine and a corresponding decrease in the free aspartate pool (Fig. 6). This pathway is especially important in marine bivalves that maintain a larger pool of free amino acids for osmotic balance, as compared to freshwater species (de Zwaan & Wijsman, 1976). The resulting oxaloacetate (OAA) is transferred to the mitochondria via the oxaloacetate-malate (OAA-MAL) proton shuttle. In anaerobically working muscle mitochondria, OAA is then hydrolyzed to fumarate and reduced to succinate via citric-acid cycle backward reactions. Zurburg and Kluytmans (1980) showed that the alanine-producing and aspartate-consuming pathways are most active in *M. edulis* adductor muscle and mantle tissue, typical for muscle cells that also constitute part of the mantel tissue. Earlier, Collicut and Hochachka (1977) demonstrated that the aspartate transamination of pyruvate fuels anaerobic ATP production in the heart ventricle of the giant oyster *Crassostrea gigas.* Experiments with radiolabeled metabolites showed that glycogen (^{14}C-glucose) administration leads to alanine labeling, whereas injection of aspartate-^{14}C (and, to a very minor extent, glutamate-^{14}C, which is not readily taken up by heart muscle cells) leads to formation of radiolabeled succinate. Both pathways are linked through the transamination reaction, which involves glutamate as an intermediary NH_2-donor (glutamine) and is catalyzed by glutamic-oxalacetic transaminase (GOT, EC 2.6.1.1, also known as aspartate transaminase; Fig. 6). Importantly, both pathways together stabilize the heart muscle redox balance with 2 moles of NADH being produced during the fermentation of 1 mole of glycogen-glucose to alanine, whereas 2 moles of oxidized redox equivalents are produced as aspartate is deaminated to OAA and oxidized to succinate (Collicutt & Hochachka, 1977).

Neither alanine nor succinate is lost from the anaerobic heart muscle cells. In the other tissues, succinate is converted to the organic acids propionate and, to a minor extent, acetate via mitochondrial pathways that generate additional ATP for anoxic survival. Both short-chained organic acids are released to the hemolymph and excreted into the surrounding seawater as long as the mussels are submersed in oxygen-free water. In air-exposed mussels the hemolymph becomes acidic and is buffered by

calcium carbonate dissolving from the shell (Zurburg & Kluytmans, 1980).

The citric-acid cycle intermediate 2-oxoglutarate (also termed α-ketoglutarate) is involved in this transamination step, which is catalyzed by aspartate transaminase (Fig. 6). It constitutes an important link between the oxygen-dependent metabolic pathways and hypoxic gene transcription, as it supports the activity of prolyl-hydroxylase (PHD) enzymes: 2-oxoglutarate deoxygenases, which also require Fe^{2+} and ascorbate to deactivate the hypoxia-inducible factor (HIF) under aerobic conditions (Koivunen & others, 2007). As long as cellular oxygen levels are high enough to support respiration, proton equivalents are transported into the mitochondrial matrix via the malate-aspartate shuttle, a membrane antiporter that transports one molecule of malate into the mitochondrial matrix for each molecule of 2-oxoglutarate it exports (Fig 6). The 2-oxoglutarate then enables the oxygen-dependent breakdown of HIF by the PHDs and, in so doing, constitutes an important metabolic signal that abrogates hypoxic gene transcription. It is characteristic of a signaling molecule that its concentration in the tissues is always kept much lower than the concentrations of other, non-signaling metabolic intermediates (Collicutt & Hochachka, 1977).

HYPOXIC, ANOXIC, AND SULFIDE-INDUCED GENE REGULATION IN BIVALVES

The hypoxia inducible factor (HIF) pathway of cellular oxygen sensing was initially identified and described in mammalian cancer cell lines, which can be exposed to chronic hypoxic stress during tumor growth. HIF is by now recognized as a master regulator of hypoxic gene transcription, a transcription factor that, in mammalian cells, induces expression of hemopoiesis genes such as the red blood cell hormone erythropoietin, angiogenesis factors, genes involved in anaerobic metabolism, glucose transporters and many others (Semenza, 2001; Fandrey, Gorr, & Gassmann, 2006; Hoogewijs & others, 2007). HIFα, the oxygen-regulated subunit of the heterodimer HIF, is structurally highly conserved throughout the animal kingdom. Activation of HIF pathways under experimental hypoxia and thermal stress was also observed in different tissues of hypoxia-tolerant fish (Heise & others, 2006; Rissanen & others, 2006), which basically confirmed its role in hypoxic gene transcription in aquatic ectotherms. However, in bivalves and more generally in mollusks, for which anoxia is a well-tolerated condition, the importance and implication of HIF expression during anoxic episodes is still largely unresolved. One possibility is that the oxygen-dependent HIF protein subunit (HIF-α) could be constitutively present, possibly with subtle fluctuations (escaping current accuracy of molecular detection) in response to the acute state of (tidal or other stress) exposure. In this case, anaerobic target-gene transcription may be controlled at the level of transactivation (nuclear translocation, HIF dimer formation, and DNA binding) in support of a constitutive contribution of anaerobic pathways to bivalve metabolism. Another possibility is that HIF in mollusks might exclusively fulfill other functions related to ontogeny and, possibly, energy compartmentalization between growth and reproduction. It is an open question, and present knowledge mostly covers the level of HIF-α mRNA transcription, which is not up-regulated during anoxic exposure but instead induced during the subsequent reoxygenation phase in the oyster *Crassostrea virginica* (Ivanina, Sokolov, & Sokolova, 2010), as well as during recovery from hypoxia and heat shock in *Crassostrea gigas* (see Kawabe & Yokoyama, 2012). Interestingly, concerted expression of HIF-α and PHD-2 transcripts during recovery in oysters was abrogated by exposure to cadmium (Cd) as secondary stressor (Ivanina, Sokolov, & Sokolova, 2010), which the authors interpreted as disturbance of nuclear translocation of HIF-α. Notably, the Cd exposure suppressed

anaerobic metabolism and transcription of genes, such as adenylate kinase, an enzyme which conserves AEC during metabolic down-regulation, causing severe energy deficiency in the Cd-treated group compared to the hypoxia exposed control group. The study by Ivanina, Sokolov, and Sokolova (2010) impressively showed how co-exposure to Cd causes energetic deficiency during prolonged anoxia by suppressing genes involved in energy-saving mechanisms. Although the HIF-activated target genes in mollusks are all but clear in hypoxia-tolerant bivalves, the HIF pathway may indeed turn out to be instrumental in maintaining constitutive gene expression in support of such energy-saving and homeostatic mechanisms as ATP recycling (adenylate kinase) and pH homeostasis (carbonic anhydrase) (David & others, 2005). Whether HIF is also involved in activating cellular protection and stress defense mechanisms, such as antioxidants and molecular chaperones, as hypoxic conditions become more severe is currently unclear.

A more differentiated picture of the nature of low-oxygen stress and the role of population-specific adaptive history was obtained for two different populations of *Arctica islandica* using a transcriptome-based approach (Philipp, Wessels, & others, 2012). The first was from the North Sea (more precisely from the German Bight) with a recorded population-specific maximum lifespan (MLSP) of 200 years. This population is currently under environmental pressure from bottom-trawling commercial fishing and warming waters in the North Sea, with unclear effects on larval survival and local *Arctica* Schumacher, 1817, recruitment. The second was a marginal but highly reproductive population from the Baltic Sea (Kiel Bight) with a MLSP of only 40 years. The bivalves from the western Baltic face highly fluctuating habitat conditions with fast and extreme changes in temperature, salinity, and oxygen depending on hydrographic conditions (winds and currents) in the Kattegat. The experimental approach involved exposure to self-induced anoxia through deep burrowing, as well as forced hypoxia and anoxia exposure by maintaining the animals at 2 kPa pO_2 (hypoxia) and 0 kPa pO_2 (complete anoxia) for 60 hours. The gene-expression analysis (by qPCR) included HIF and PHD mRNAs, antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), HSP70, and the metabolic markers malate dehydrogenase (MDH) and opine dehydrogenase (ODH). In the Baltic Sea population, the majority of these stress-induced genes were up-regulated in response to anoxia but not hypoxia, but the induction could be completely reversed when bivalves were left to recover up to 6 hours at normoxic conditions. Also, the mRNA of the hypoxia-inducible transcription factor HIF increased in hypoxia and anoxia and was low again after 1 hour and 6 hours of normoxic recovery. Note that in mammals HIF is constitutively expressed and regulated at the protein level through proteasomal breakdown after oxygen-dependent hydroxylation by the prolyl hydroxylases. In contrast to this pronounced stress response, transcription of all genes was down-regulated in North Sea individuals during hypoxic exposure, compared to normoxic controls, and this down-regulation was even stronger in anoxia. Furthermore, deep burrowing caused the same down-regulation of stress-induced genes in North Sea *Arctica*. The authors concluded that anoxia, but not hypoxia, triggers a stress response in Baltic Sea specimens, and they related this to stress hardening (sublethal stress that conditions individuals in natural populations to mount a pronounced stress response under severe stress exposure) through adaptation to constant abiotic fluctuations. However, the physiological disposition to mount a pronounced stress response appears to be at the cost of cellular maintenance and longevity and possibly explains the shorter lifespan in the Baltic population (Philipp, Wessels, & others, 2012). The accumulation of fluorescent age pigments, indicative

of the velocity of cellular aging, is much faster in the Baltic Sea population, and 30-year-old Baltic Sea individuals have a much higher age-pigment (lipofuscin granule) density than North Sea or Icelandic *Arctica islandica* centenarians. Exposure to hypoxia and subsequent reoxygenation may produce hazardous reactive oxygen species, and the response in the Baltic *Arctica* can be interpreted as an anticipatory response to suppress oxidative damage, although activity measurements of CAT and GPx in the North Sea and Baltic experimental groups did not yield consistent results (CAT activities diminished in all groups and GPx activities were slightly enhanced in North Sea and Baltic Sea specimens under hypoxic and anoxic exposure compared to controls). No hypoxia or anoxia endurance or LT_{50} analyses were performed in this study, but the pronounced anoxia tolerance of Baltic Sea *Arctica islandica* is widely known (Theede, 1973).

Seasonal differences of resistance to moderate hypoxia (10 kPa pO_2) and normoxic hydrogen-sulfide exposure were recorded in short-lived coquina clams, *Donax variabilis* Say, 1822, residing in the sediment surface of high-energy beaches, where they are unlikely to encounter either hypoxia or hydrogen sulfide (Joyner-Matos, Downs, & Julian, 2006). Hypoxia LT_{50} was five days in spring and two days in fall; in neither case were stress genes induced under these mild stress conditions. On the contrary, hydrogen-sulfide stress (0.1 mmol L^{-1}) applied in fully oxygenated water caused a major induction of oxidative stress-induced genes (including antioxidants and the mitochondrial DNA repair enzyme OGG1-m), and the authors consequently concluded that the animals died from oxidative stress caused by the H_2S exposure. Indeed, oxic hydrogen-sulfide exposure may even be the problem, as aerobically working mitochondria can become allosterically inhibited by H_2S, a situation bound to cause a spill of ROS from the autoxidation of reduced respiratory chain components (e.g., ubiquinone). Thus, only a strong stressor such as H_2S that potentially causes complete blockage of mitochondrial electron flow, similar to anoxia, could eventually trigger the stress response.

CELLULAR RESPONSE TO PHYSICAL STRESS OF HIGH TEMPERATURE AND HYPOSALINITY: GENE EXPRESSION AND ANTIOXIDANT PROTECTION

Burrowing clams and mussels are masters among facultative anaerobes and, as seen before, low tissue-oxygen levels, metabolic depression, and even some level of anaerobic energy production are not only tolerated, but represent the optimal physiological status in these animals (Abele & Philipp, 2012; Joyner-Matos & others, 2007). Stress results when aerobic respiration is entirely blocked or when the additional tissue oxygen demand—induced by (critically) high temperature, salinity shock, or toxic insult—exceeds the oxygen delivery to central tissue regions (Pörtner, 2001; Sokolova, Sukhotin, & Lannig, 2012). Strong fluctuations of tissue PO_2 (hypoxia-reoxygenation) cause ROS production, and oxidative stress is, therefore, technically the central cellular phenomenon in the response to a plethora of stressors.

HIGH-TEMPERATURE STRESS

Intertidal and shallow-water bivalves are regularly exposed to high-temperature stress, often in combination with desiccation during low tides and hypoxic stress from shell closure. Most ex-situ experimental approaches to date have considered only single stressors to provide a mechanistic understanding of the nature of the cellular stress response.

Experimental exposure of marine bivalves to high temperatures has been shown to enhance oxidative stress, mostly confirmed by enhanced accumulation of lipid peroxidation biomarkers, such as thiobarbituric acid reactive substances (TBARS). As in all other groups of marine ectotherms, the response in different species or populations

is strongly shaped by climatic preadaptation. Acute warming of temperate soft-shell clams, *Mya arenaria,* from the Wadden Sea, the intertidal zone of the southeastern North Sea, from 10°C (control temperature) to 18°C and 25°C (stress temperature) caused slightly enhanced TBARS levels in mantle tissue, although the activities of both antioxidant enzymes, superoxide dismutase (SOD), and catalase were increased at both stress temperatures (Abele & others, 2002). Similarly, fourteen days of warming from 26°C (control temperature) to 32°C (stress temperature) caused elevated TBARS concentrations and induced the activities of antioxidant enzymes SOD, catalase, GPx, glutathione reductase (GR), and glutathione-s-transferase (GST) in the tropical mussel *Perna viridis* (Linnaeus, 1758 in 1758–1759) (Verlecar, Jena, & Chainy, 2007). In comparison, stenothermal Antarctic mollusks are highly adapted to a permanently cold climate and have very narrow windows of temperature tolerance, within which they can compensate for minor heat stress. The Antarctic protobranch *Aequiyoldia eightsii* (Jay, 1839), only tolerates warming to +2°C. Exposure to heat above this temperature caused a sharp rise of respiration rate to combat extreme and finally lethal heat stress (>5°C), while SOD activities diminished and TBARS levels increased massively during the incubation above the critical temperature (Abele & others, 2001). In the Antarctic stenothermal limpet *Nacella concinna* (Strebel, 1908), catalase was nearly constant, whereas SOD activity was elevated at tolerance temperature of 4°C (compared to 0°C control temperature) but collapsed under extreme heat exposure (9°C) and respiration rates also declined (Abele & others, 1998). These experiments show that antioxidant activities form part of the response to mild heat stress in bivalves and other mollusks and their decrease is a good indicator of the thermal limit at which survival is critically compromised.

More detailed knowledge of gene induction in marine gastropods and bivalves exposed to heating and other forms of stress is now available. In a biogeographic study, comparing constitutive heat-shock protein (Hsp) expression and inducibility of the heat stress response during air exposure on the shore line, Koenigstein and others (2013) confirmed the importance of climatic preadaptation in Antarctic limpets, *Nacella concinna,* from King George Island (South Shetland Islands) and two closely related limpets from the sub-Antarctic (Punta Arenas) and cold-temperate (Puerto Montt) climate zones in coastal Chile. Whereas the constitutive Hsp levels were similar in all three limpet species, the inducibility was highest in the northernmost *Nacella magellanica* (Gmelin, 1791) from Puerto Montt, which were adapted to the warmest and most changeable climate at the northern edge of their distribution. Other recent papers also compared antioxidant gene induction to changes in enzyme activities or protein levels. In gills of the disk abalone *Haliotis discus discus* (Reeve, 1846), several antioxidants were consistently (CAT, GPx, and the H_2O_2 decomposing factor TPx) or transiently (e.g., mitochondrial MnSOD) upregulated during a 24-hour exposure to stressfully high temperature in support of antioxidant activities under heat stress (De Zoysa & others, 2009). A comprehensive study of heat stress was carried out by Park and others (2008b), in which the cold-stenothermal Antarctic clam *Laternula elliptica* was exposed for four days to a critically high temperature of 10°C. The animals studied were again collected at King George Island at the northern tip of the West Antarctic Peninsula, a region that has undergone rapid, climate-change–driven warming over the past decades. The bivalves were collected from shallow-water depths of ~20 m (Park & others, 2008b). Thermal tolerance of these clams had been tested previously at Rothera Station (located further south on the peninsula) and showed 50% mortality after seven days at 9°C (Peck,

Portner, & Hardewig, 2002), for which reason Park and others (2008b) limited their experiment to four days of heat exposure. The authors measured antioxidant enzyme activities, glutathione levels, and protein oxidation as markers of oxidative stress in the polar clam and saw only a slight, transient increase of SOD (but not CAT) activity in gills and the digestive gland on day 1, before oxidative damage of proteins started to become visible in both organs starting on day 2. While the basal antioxidants SOD and CAT succumbed to the heat stress, the concentration of the redox buffer glutathione (GSH) increased massively in both tissues in connection with glutathione-related enzyme activities after two days of exposure (Park & others, 2008b). Furthermore, two different peroxiredoxin genes were transcriptionally induced—enzymes that detoxify H_2O_2 and alkyl hydroperoxides and are generally involved in controlling cellular ROS levels and ROS signals (Park & others, 2008a). This kind of redox buffering and ROS control seems to be the ultimate attempt to suppress thermally induced ROS damage under life-threatening heat stress. Park, Ahn, and Lee (2007) also obtained a full-length Hsp70 sequence for *L. elliptica* and observed a significant increase in Hsp70 gene-expression levels in the gills and digestive gland of heat-stressed (10°C) animals, indicating that 20 Ma of evolution in cold climate has by no means abrogated the heat-shock response in this stenothermal bivalve, as had been suggested for some Antarctic fish species (Hofmann & others, 2000).

The heat-shock response centrally involves the induction of Hsp to prevent the formation of cytotoxic aggregates in cells. Hsps are molecular chaperones that stabilize unfolded and salvage denatured proteins under various stress conditions. Inducible Hsps are upregulated under all kinds of stress that cause protein damage, including heat stress and oxidative stress, and play a role during apoptosis and bacterial infections in mollusks (Wang, Yang, & Song, 2013). Tanguy and others (2008) detected Hsp 70, Hsp 90, and Hsp 40 in bivalve expressed sequence tag libraries (e.g., in *Mytilus edulis*), and Wang, Yang, and Song (2013) found that Hsp70 variants, in particular, are expressed by mytilids, oysters, and various clam species (see Table 1 for list of full-length Hsp70 sequences available for molluscan species). Tissue levels of inducible and constitutive Hsps change with seasonal temperature acclimation and, in summer, basal tissue levels of Hsp70 mRNA are higher while, on the other hand, the threshold temperature for stress-controlled induction rises (Hofmann, 1999). Regulation of constitutive and inducible Hsp expression is under the control of different transcription factors, of which the heat-shock factor 1 pathway is the central operator that activates Hsp transcription by interacting with the heat-shock elements in the promoter region of the Hsp genes (for review, see Wang, Yang, & Song, 2013). The signaling cascade for the induction of Hsp70 (through heating from 15°C to 30°C) in *Mytilus galloprovincialis* from the Mediterranean involves phosphorylation of p38-MAPK (mitogen-activated protein kinase) or JNK (c-Jun N-terminal kinase), as shown by Gourgou and others (2010), who used specific inhibitors for both transcription factors in their experiments. Phosphorylation of both kinases enables their translocation to the nucleus where they activate gene transcription through phosphorylation of such target transcription factors as c-Jun, ATP family proteins, NFκB, or Elk, which then induce the heat-shock response. It is interesting to note that the activities of the basal antioxidants (CAT, SOD, GST) were hardly affected by heat stress in *M. galloprovincialis*, while transcription of all three genes was activated at 22°C and 24°C within one day of heat exposure and returned back to control levels after three days of heat stress (Banni & others, 2014). Cross-exposure to toxic Ni and heat exacerbates the oxidative stress response in the Mediterranean mussels.

The comprehensive study by Tomanek and Zuzow (2010) compared the response to heat stress of the Californian mussel

Mytilus trossulus Gould, 1850, and Mediterranean *M. galloprovincialis* at the level of protein expression. The latter has been introduced to the Californian coast by bilge water transport and is replacing the less heat-tolerant *M. trossulus* at its southern distribution limit. In this study, they heated both mussels from 13°C to 24°, 28°, and 32°C and measured the expression levels of numerous functional proteins by 2D-GE using mass spectrometry for peptide fingerprinting. They found evidence for a complex heat-shock response in both animals, but no pronounced induction of antioxidant proteins (thioredoxin, aldehyde dehydrogenase, and one CuZnSOD isoform) occurred. Instead, several cytoskeletal proteins were enhanced in both bivalves in combination with molecular chaperones (Hsps) and proteasome subunits, which the authors interpreted as response to cytoskeletal damage by enhanced salvage and repair mechanisms. Furthermore, key proteins of the electron-transport system, nicotinamide adenine dinucleotide phosphate (NADH) oxidoreductase and cytochrome-c reductase, as well as of the citric-acid cycle, pyruvate dehydrogenase (PDH) and mitochondrial malate dehydrogenase (MDH), were downregulated, indicating a reduction of NADH-consuming aerobic metabolism and the concomitant ROS production at high temperatures. On the other end, enzymes of the pentose-phosphate cycle (transketolase and lactonase) and mitochondrial isocitrate dehydrogenase (IDH) were up-regulated inducing NADPH production which supports antioxidant mechanisms in cycling oxidized GSSG to GSH (Tomanek & Zuzow, 2010). In addition, one sirtuin isoform (NAD-dependent-deacetylase) was down-regulated under heat stress, which may reduce life expectancy in heated mussels. Based on their molecular analysis, Tomanek and Zuzow proposed three possible central effects of heat injury in bivalves: (1) disintegration of the cytoskeleton; (2) overproduction of toxic ROS that leads to major metabolic reorganization involving down-regulation of NAD-producing pathways and up-regulation of NADPH-producing pathways but not necessarily leading to enhanced quantities of the classical antioxidants; and (3) reduction in lifespan through down-regulation of sirtuins. This third effect is interesting as it may contribute to the prevention of stress-induced metabolic depression under stressful heat exposure, which exacerbates the critical situation. In that case, signaling for a shorter lifespan can lead to gonad spending to support a new generation of mussels before the parent generation succumbs to lethal stress.

As transcriptomics and proteomics become more accessible and new tools also become available for non-model species, several authors have analyzed the response to different stressors or stressor combinations in molluscan models. De Zoysa and others (2009) observed a differentiated response in the transcription of antioxidant and immune-competence genes to heat stress in disk abalones; however, in response to hyposaline conditions (32–25 psu [practical salinity units]) for 24 hours, all tested stress-induced genes were consistently up-regulated. Exposure of the Mediterranean *M. galloprovincialis* to a suite of lower salinities—between 37 psu and 11 psu—caused a tiered stress response, including reduced fitness, increased respiration rates, and DNA oxidative damage, as well as increasing phosphorylation of MAPK at lower salinities (Hamer & others, 2008). Generally, the mussels were more susceptible in summer than in winter to low salinity stress. Expression of metallothioneins, small proteins that limit oxidative stress by binding transition metals, was reduced at lower salinities in summer-collected animals. Only under detrimental stress at 11 psu, which caused increased mortality, were these stress markers highly expressed. Tomanek and others (2012) exposed the Californian mytilids *M. trossulus* and *M. galloprovincialis* to 4 hours of hyposaline shock—from 35 psu (control) to 29.8 psu and 24.5 psu—followed by a 24-hour recovery at control salinity and

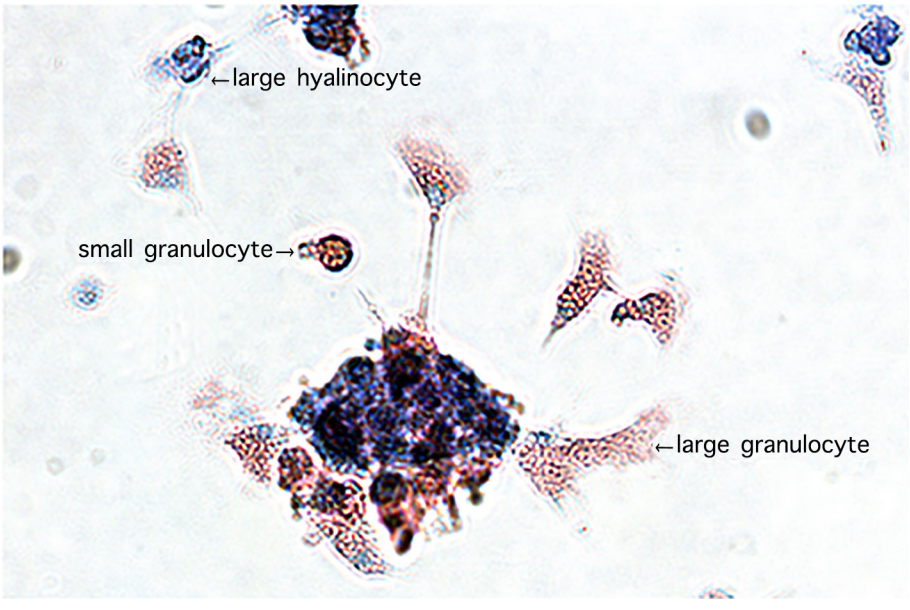

Fig. 7. May-Grünwald/Giemsa staining of *Mytilus edulis* (Linnaeus, 1758 in 1758–1759) hemocytes, showing small and large granulocytes (stained red), as well as large and small hyalinocytes (*stained purple*) (new; photograph by J. Saphoerster, Institute of Clinical Molecular Biology Kiel, Germany).

again measured the response at proteome level. Major up-regulation of small Hsps (e.g., GRP78 and GRP94) indicated protein unfolding in the endoplasmatic reticulum subcellular compartment during hyposaline exposure. Proteasome subunits were less enhanced than under heat stress (see above), and the authors proposed a general slow-down of protein turnover under hyposaline shock. Again, energy metabolism enzymes from NADH-producing cellular pathways (PDH, MDH, ATP-synthase, IDH) were down-regulated under hyposaline shock and 1 day of recovery, as were some major antioxidant enzymes, indicating some level of metabolic-rate depression and reduction of ROS levels (Tomanek & others, 2012). Meng and others (2013) exposed oysters during seven days to a variety of salinities from 5 psu to 40 psu (at 5 psu intervals) and used a transcriptomic approach and qPCR to analyze the cellular response patterns and find marker genes for these different salinity-stress conditions. They found that seven days of low (10–15 psu) and also high (40 psu) salinity stress decreased the expression of voltage-gated Na^+/K^+ channel genes, which results in membrane depolarization and is adaptive to the lower salinity. On the other hand, they found that expression of the Ca^{2+} channels was increased, which alters the Ca current. They also observed that expression of aquaporins (water channels) was reduced in low- and high-salinity treatments to prevent shrinking or swelling of gill cells. Furthermore, they analyzed and mapped the changes occurring in the free amino-acid pool in salt-stressed oysters. Important findings in the response to low-salinity stress included the up-regulation of alanine transaminase that converts pyruvate to alanine and could also be involved in metabolic slowdown and anaerobic metabolism (Fig. 6). Spermidine synthase and aldehyde dehydrogenase (ALDH), two enzymes involved in alanine formation based on arginine and proline metabolism, were also up-regulated in hyposalinity, ALDH with its antioxidant/reducing properties showing the same response to low salinity

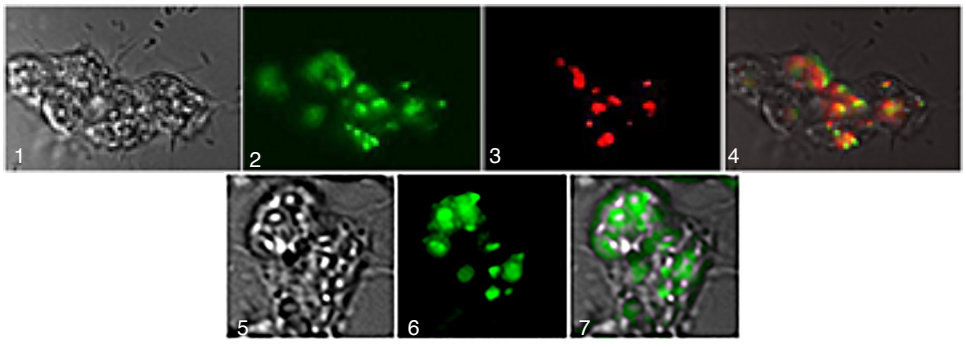

FIG. 8. Reactive oxygen species production in *Mytilus edulis* (LINNAEUS, 1758 in 1758–1759) hemocytes upon immune stimulation by zymosan particles (*green fluorescence*) or *Vibrio anguillarum* (BERGEMAN, 1909) MACDONELL & COLWELL, 1985 (*red fluorescence*); *1, 5,* transmission; *2–3, 6,* fluorescence; *4, 7,* overlaid images are shown in this order from left to right. (new; photograph by J. Saphoerster, Institute of Clinical Molecular Biology, Christian-Albrechts Universität-Kiel).

as in mussels (TOMANEK & others, 2012). Another antioxidant up-regulated in hyposaline stress is GPx (glutathione peroxidase), which scavenges H_2O_2 produced in the cells when exposed to low salinity. Thus, also in low-salinity stress, some central pathways are induced that include genes for osmoregulation and ROS-reducing mechanisms.

IMMUNE SYSTEM AND RESPONSE TO ENVIRONMENTAL STRESS

As discussed above, bivalves experience a multitude of environmental stress conditions related to climate change. Other stress inducers are anthropogenic pollution or invading species. To survive the different forms of stress, bivalves need an effective immune system to control and steer the interactions with microbes and pathogens within a given environmental setting. Recognition of self and nonself and the maintenance of self-integrity are pivotal elements for survival and proper physiological function. To consistently achieve and maintain such integrity, bivalves have evolved an efficient immune system. This surveillance system recognizes nonself structures such as microorganisms, viruses, and other foreign particles, and it also detects and eliminates modified self-cells (e.g., cancer cells or damaged and destroyed cells). Bivalves rely exclusively on the innate immune system encoded in the germ line and lack the somatically rearranged, adaptive immune system that is found in vertebrates. The vertebrate adaptive immune system recognizes and memorizes newly invading pathogens, targets the immune response, and enhances protection under reinfection. Despite these great achievements, the adaptive immune system is not yet able to function on its own, relying strongly on the innate immune system as a first line of defense and to mediate further adaptive immune responses (MEDZHITOV & JANEWAY, Jr., 1998). The innate immune system consists of physiological barriers as well as cell-mediated immune responses, such as phagocytosis (Fig. 7) or ROS production (Fig. 8) by immune cells. Even though the innate immune response is not specific for a particular pathogen, it is directed against a broad spectrum of invaders and responds much faster than the adaptive immune response.

As outlined above, most bivalves are filter- and deposit-feeders that constantly engulf bacteria and other microorganisms. In bivalves, bacteria constitute an important food source and, just like other marine invertebrates, bivalve tissues are coated by a biofilm of bacterial symbionts (RUBY & MCFALL-NGAI, 1999; DUBILIER, BERGIN, & LOTT, 2008). Unlike these

mostly beneficial bacteria (which are sometimes even necessary for survival), pathogenic bacteria can cause mass mortalities of marine invertebrates (Le Gall & others, 1988; Gómez-León & others, 2005). Thus, control and elimination of these bacteria by the host is of vital importance to ensure optimal physiological function.

The innate immune system performs several major tasks. These include a controlled host-microbial crosstalk at epithelial barriers, the recognition of danger signals, the recruitment of mesoderm-derived professional immune cells, the clearance of intracellular pathogens by phagocytosis with the aid of ROS and reactive nitrogen species (RNS) such as NO and peroxynitrite, and the secretion of local or circulating effector-molecules (such as antimicrobial peptides and simple opsonic forms of complement).

In bivalves, hemocytes represent the major migratory cell type of the innate immune system. Different hemocyte subpopulations are differentially involved in the host's biological and physiological functions, including nutrient transport and digestion, tissue and shell formation, maintenance of homeostasis, and immune response (Mount & others, 2004; Canesi & others, 2006). However, the main function of bivalve hemocytes is believed to be the immune response. Hemocyte receptors can bind to surface molecules directly or via mediating adapter-molecules (opsonization effects), 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 & others, 2002). The humoral response includes permanent or inducible biosynthesis of antimicrobial peptides or enzymes (Bettencourt & others, 2007). Cellular reactions such as phagocytosis or encapsulation of particles are mediated by the hemocytes (Cheng, 1975; Malham & others, 2003) .

HEMOCYTES, PATTERN RECOGNITION, AND PHAGOCYTOSIS

Bivalve hemocytes can be divided into agranular (hyaline, hyalinocytes) and granular (Fig. 7). The latter are further differentiated into eosinophilic and basophilic cells (Canesi & others, 2002; Wootton, Dyrynda, & Ratcliffe, 2003).

These cell types react in number and response to different stress scenarios (e.g., temperature and salinity changes and wound healing), and are most important for the recognition, elimination, and control of microorganisms and other foreign particles. One of the major enigmas of the immune system, which also relates to one of the greatest differences between adaptive and innate immunity, is how organism can distinguish normal healthy cells from foreign or abnormal, endogenous cells. To distinguish self from nonself, the adaptive immune system uses the antigen-antibody reaction, while the innate immune system mainly relies on germ-line encoded pattern recognition receptors (PRRs).

The PRRs sense invariant molecular signatures that are either present in microorganisms (microorganism-associated molecular patterns) or pathogens (pathogen-associated molecular patterns). For example, lipopolysaccharides or unmethylated CpG (linear cytosine-guanine dinucleotide) DNA can signal the presence of nonself particles. Alternatively, PPRs can recognize signals derived from endogenous sources that indicate profound cellular damage (e.g., extracellular heat-shock proteins, oxidatively modified proteins). The major types of PRRs in the innate immune system are the Toll-like receptors (TLRs), peptidoglycan recognition receptors (PGRPs), gram-negative binding proteins (GNBPs), the intracellular NOD-like receptor (NLR), RIG-like receptors (RLR), and the family of scavenger-receptor cysteine-rich (SRCR) domain-containing proteins (Sarrias & others, 2004). TLRs, PGRPs, and GNBPs are PRRs which recognize bacteria, fungi, and viruses on the

cellular surface. NLRs and RLRs play a major role in the recognition of intra-cellular bacteria and viruses, respectively.

Due to the large distribution of and variety in ecological niches, it is not surprising to find diverse immune responses including recognition receptors in bivalves. The recent advances of high-throughput sequencing technology and, with these, whole genome and transcriptome analysis of non-model organisms, have accelerated the identification of orthologs for known immune genes (such as PRRs and the discovery of a whole suite of new immune and defense responses) from other organisms (Venier & others, 2003; Rosenstiel & others, 2009; Philipp, Kraemer, & others, 2012). High sequence variety has been found within different PRR families in bivalves, which points towards a complex suite of recognition processes (Philipp, Kraemer, & others, 2012; Yuen, Bayes, & Degnan, 2014). Further functional studies are now aimed at understanding the specificity of PRRs and downstream pathways in bivalve hemocytes.

A major mechanism of the hemocyte immune response after recognition of foreign particles is phagocytosis, which is linked to the secretion of humoral defense factors such as agglutinins (e.g., lectins), lysosomal enzymes, antimicrobial peptides, and complement factors, as well as the release of ROS and RNS (Roch, 1999; Canesi & others, 2002; Tahseen, 2009). Phagocytosis occurs by invagination of the cell membrane and the formation of an endocytic vacuole, called the primary phagosome. Cytoplasmic granules containing phosphatases, esterases, amidases, and oxidative enzymes such as peroxidase and cytochrome c oxidase then migrate and fuse with the phagosome to form a secondary phagosome, which degrades and eliminates the foreign particles (Donaghy & others, 2009). Lysosomal enzymes such as lysozyme can be found within the cell, but they are also present in extracellular forms in the hemolymph and participate in the killing and degradation of foreign organisms by ROS, generated by the immunocyte, a process called oxidative burst (Lopez & others, 1997). Thus, in certain ways, hemocytes resemble the vertebrate monocyte-macrophage lineage for which the cellular response of ROS generation (oxidative burst) has been described.

ROS AND RNS GENERATION BY IMMUNE CELLS

Oxidative (or respiratory) burst is the terminology used for humans and other vertebrates and describes the massive release of ROS over a short time period, coupled with a high oxygen uptake (Broeg & Steinhagen, 2012). It is a key reaction during phagocytosis, carried out by macrophages and neutrophils to degrade bacteria or internalized harmful substances.

In vertebrates, the cascade of enzymatic reactions starts upon activation of NADPH-oxidase complexes (NOX) with the production of the superoxide anion ($O_2^{\cdot-}$). Hydrogen peroxide (H_2O_2) can then be produced from superoxide either spontaneously or catalysed by SOD. In the presence of reduced transition metals, H_2O_2 can further be converted into the highly reactive hydroxyl radical (·OH). Hydrogen peroxide may also give rise to highly toxic hypochlorous acid (HOCl), a potent antimicrobial oxidant (Klebanoff, 2005), catalysed by myeloperoxidase in the presence of chloride ions, as described for the mammalian system (Klebanoff, 2005) and fish (Castro & others, 2008).

In bivalves, ROS generation of hemocytes was extensively investigated and is also often termed oxidative burst, but it is generally less intensive and proceeds more slowly than in vertebrates. Peak ROS generation was found to take place after 15 to 70 minutes, and the intensity is 2 to 10 times higher than the control levels of ROS formation in hemocytes. In vertebrates, however, ROS generation reaches values up to 1000 to 1500 times higher than the basis levels and only lasts a few minutes (Donaghy & others, 2009). Thus, oxidative burst is a term that should be used with caution for the molluscan immune response.

The amount of hemocyte-derived ROS varies among different bivalve species and according to the stimulant used to elicit the response (Moss & Allam, 2006; Wang & others, 2008; Donaghy & others, 2009). Common stimulants are lipopolysaccharide (LPS) and peptidoglycan (PGN), which are important structural constituents of the outer membrane of gram-negative (LPS, PGN) and gram-positive (PGN) bacteria. Flagellin is a main component of bacterial flagellar filaments. Zymosan, a protein-carbohydrate-complex derived from yeast cell walls, is widely used as experimental inducer of inflammation or phagocytosis. In most bivalve species—including oysters such as *Crassostrea virginica, C. gigas, C. ariakensis,* and *Ostrea edulis* (Linnaeus, 1758 in 1758–1759); mussels such as *Mytilus edulis* and *M. galloprovincialis;* and scallops such as *Pecten maximus* and *Chlamys farreri* (Jones & Preston, 1904)—hemocytes generate ROS activated by one of these stimulants (for review, see Donaghy & others, 2009). Zymosan seems to be a potent stimulator of ROS generation; in most studies of different species and phylogenetic groups (Bivalvia, Crustacea), hemocyte activation could be elicited with this stimulant (Fig. 8).

Different types of hemocytes (e.g., agranular and granular) have a different potential to produce ROS. In *Mytilus edulis,* a higher capacity for respiratory burst was found for eosinophilic compared to basophilic granulocytes (Pipe, Farley, & Coles, 1997). In *Crassostrea virginica*, all types of hemocytes (i.e., granulocytes, hyalinocytes, and small granulocytes) showed increased production of ROS in the presence of zymosan, with granulocytes showing the highest increase (Hégaret, Wikfors, & Soudant, 2003). Generally, granulocytes seem to exhibit a higher ROS generation potential than hyalinocytes (Hégaret, Wikfors, & Soudant, 2003; Labreuche, Lambert, & others, 2006; Labreuche, Soudant, & others, 2006).

The respiratory burst in bivalve hemocytes is modulated by factors of the hemolymph. In *Mytilus* Linnaeus, 1759 in 1758–1759, 17ß-estradiol stimulates oxyradical production (Canesi & others, 2006). Also, ROS generation of *Mytilus* hemocytes stimulated with *Vibrio parahemolyticus* and *E. coli* is enhanced in the presence of plasma (Kumazawa, Morimoto, & Okamoto, 1993). However, the mechanism underlying these modifications has not been elucidated. Background ROS generation can be detected in non-stimulated hemocytes, which may demonstrate low ROS production during normal metabolism (Pourova & others, 2010). Furthermore, isolation procedures or the attachment of the hemocytes to the experimental surface (glass, plastic) might lead to activation and ROS generation (Anderson, 1994; Moss & Allam, 2006).

In addition to immunocytes, the generation of ROS can also result from the prophenoloxidase system in the hemolymph, which has been observed in several invertebrates, including bivalves (Cerenius & Söderhäll, 2004; Cerenius, Lee, & Söderhäll, 2008). The main function of this system is the synthesis of melanin, which physically shields the host from invading pathogens. Quinone intermediates that are generated during melanin synthesis can enter enzymatic and non-enzymatic redox cycling, producing the corresponding semiquinone radicals and, as a result, produce $O_2^{\cdot}$ (Bogdan, 2007).

The massive release of ROS due to infection can be both beneficial and harmful. On the one hand, ROS can help degrade foreign particles; on the other hand, high amounts of ROS can lead to imbalance in the cellular antioxidant defense, resulting in oxidative stress. Antioxidants like the enzymes SOD and catalase, as well as enzymes of the glutathione system, glutathione peroxidase and reductase, have been detected in the hemolymph and hemocytes of bivalves (Li & others, 2008), and these enzymes may serve as a protective mechanism to control ROS concentration and avoid oxidative damage.

Hemocytes of marine bivalves release NO in addition to ROS, as described in studies

of *Mytilus edulis* (Ottaviani & others, 1993; Canesi & others, 2006), *M. galloprovincialis* (Arumugam & others, 2000; Gourdon & others, 2001; Tafalla, Novoa, & Figueras, 2002), and *Ruditapes decussatus* (Linnaeus, 1758 in 1758–1759) (Tafalla & others, 2003). In vertebrates, various cells involved in the general immune response are known to release NO, including monocytes, macrophages, neutrophils, eosinophils, but also epithelial cells, fibroblasts, and hepatocytes. RNS production is catalyzed by the enzyme NO synthase—endothelial NOS (eNOS), inducible NOS (iNOS), neuronal NOS (nNOS)—and starts with the production of the NO radical (NO·) via the transformation of l-arginine. NO was found to inhibit the adhesion of platelets and leukocytes to the endothelium and also to influence leucocyte recruitment and adhesion, as well as chemotactic response and the production of chemokines (Oliveira, Silva-Teixeira, & Goes, 1999). NO can be converted by reaction with O_2·, $RO^{\cdot}$, and $RO_2^{\cdot}$ to various other RNS, such as nitrosonium cation (NO+), nitroxyl anion (NO·), or peroxynitrite (ONOO·). Peroxinitrite is a very powerful oxidant, 2000 times more potent than H_2O_2 in oxidizing thiols, and can also decompose to HO·, the short-lived and also very toxic hydroxyl radical (Freeman, 1994; Victor & others, 2005).

In bivalves, both NO and other RNS have important microbicidal effects against intracellular parasites, bacteria, fungi, and protozoa (Arumugam & others, 2000; Reeves & others, 2003). Although $O_2^{\cdot}$ is, in itself, of low bactericidal activity, once it dismutates to H_2O_2 it can generate highly bactericidal NOO· through a reaction with NO (Reeves & others, 2003).

Several pathogens have found a way to avoid destruction by the oxidative burst system. Bacteria such as *Staphylococcus aureus* (Mandell, 1975) and parasites like *Plasmodium berghei* (Fairfield, Eaton, & Meshnick, 1986) can avoid death from oxidative burst through the action of antioxidant enzymes (e.g., catalase). Different pathogenic bacterial strains are known to suppress ROS generation of bivalve hemocytes (Lambert & Nicolas, 1998; Labreuche, Lambert & others, 2006). The facultative intracellular parasite *Perkinsus marinus* of the eastern oyster *C. virginica* prevents the accumulation of ROS by abrogating the synthesis and by scavenging the products of the host's phagocytic respiratory burst (Fernández-Robledo, Schott, & Vasta, 2008).

The major ROS generation sites of hemocytes are membrane-bound NADPH-oxidases (NOX, DUOX). NOX enzymes transfer electrons across membranes to reduce oxygen to superoxide and exhibit four conserved domains: a C-terminal NADPH-binding site, a FAD-binding site, six transmembrane domains and heme-binding histidins (Lambeth & others, 2000; Lambeth, 2002). ROS-generating NOX-DUOX orthologs are conserved in vertebrates, urochordates, echinoderms, nematodes, insects, fungi, plants amoeba, and red alga, but not in prokaryotes. Orthologs have been found also in marine bivalves (Saphörster, 2013), but little is known regarding the possible link between NOX-DUOX homologs and immune function in bivalves. In hemocytes of the bivalves *Mytilus edulis*, *Arctica islandica,* and *Laternula elliptica,* several orthologs of NOX and DUOX have been identified at the molecular level, and there is evidence of regulated H_2O_2 release with regard to immune function (Husmann & others, 2011; Saphörster, 2013). The link between NOX-DUOX and ROS generation is, however, still missing (Saphörster, 2013).

ANTIMICROBIAL PEPTIDES

Phagocytosis and respiratory burst represent only two parameters of the bivalve's immune repertoire. In addition to these, hemocytes are known to secrete antimicrobial peptides into the hemolymph for bacterial recognition and killing (Pruzzo, Gallo, Canesi, 2005). Antimicrobial peptides are widely expressed in invertebrates and vertebrates to defend against bacteria and fungi, either by directly killing

or slowing growth of the invaders. In vertebrates, and presumably also in invertebrates, they further act as modulators of the immune system—for example, by stimulating cytokine release or chemotaxis (Otero-Gonzalez & others, 2010). In mollusks, the occurrence and function of antimicrobial peptides has been especially well documented in *Mytilus edulis* and *M. galloprovincialis* (Venier & others, 2011; Philipp, Kraemer, & others, 2012). The antimicrobial peptides identified so far can be sorted into four groups: defensins, mytilins, myticins, and mytimycins (Mitta & others, 2000; Mitta, Vandenbulcke, & Roch, 2000). The different peptide groups show distinct antimicrobial properties against gram-positive and gram-negative bacteria and fungi.

EXTERNAL AND INTERNAL FACTORS INFLUENCING THE IMMUNE RESPONSE

External ecological factors and internal physiological status influence an individual bivalve's ability to perform a proper immune response. Under conditions affecting physiological status (e.g., starvation) or during bacterial mass-proliferations due to eutrophication or warming, pathogenic bacteria can cause mass mortalities in different bivalve species (Cheney, MacDonald, & Elston, 2000; Cao & others, 2007; Garnier & others, 2007; Allen & Burnett, 2008). 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 (Canesi & others, 2002; Auffret, 2005; Donaghy & others, 2009). A novel aspect of bivalve physiology, which links physiological stress and immune responses, is the regulation of molecular effectors such as functional peptides and proteins in hemocytes and soft-body tissues (Koutsogiannaki & Kaloyianni, 2010; Husmann & others, 2014). Proteomic and transcriptomic studies in bivalves point towards a commonly induced set of stress proteins (Tomanek, 2011). These comprise heat-shock proteins (Hsps), which were found to be involved in the stabilization of proteins, regulation of oxidative stress, repair of tissue damage, tissue development, and antimicrobial defense (Clark, Fraser, & Peck, 2008; Wang, Yang, & Song, 2013).

External factors such as food availability, mechanical disturbance, temperature, and inorganic pollution (i.e., xenobiotics) and the accompanying physiological consequences, such as starvation, injury, or imbalanced energy allocation, can modulate immune responses in bivalves (Butt & others, 2007; Hawley & Altizer, 2011; Husmann & others, 2011). Furthermore, bivalve development and aging can influence immune-system capacity, thereby altering the response to external factors. Lower numbers of free-floating hemocytes but higher basal and stimulated (zymosan) ROS generation per cell and altered gene expression of immune genes upon injury were found in younger *Laternula elliptica*, indicating individual age to be an important modulator of bivalve immune response (Husmann & others, 2011; Husmann & others, 2014).

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