EFFECTS OF MECHANICAL DISTURBANCE AND SALINITY STRESS ON BIOENERGETICS AND BURROWING BEHAVIOR OF THE SOFT SHELL CLAM *MYA ARENARIA*

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Abstract. Bioturbation of sediments by burrowing organisms plays a key role in the functioning of the coastal ecosystems. Burrowing is considered an energetically expensive activity, yet the energy costs of burrowing and the potential impacts of multiple stressors (such as salinity stress and wave action) on bioenergetics and burrowing performance of marine bioturbators are not well understood. We investigated the effects of mechanical disturbance and salinity stress on the burrowing behavior, aerobic capacity and energy expense of digging in a common marine bioturbator, the soft clam *Mya arenaria* from the Baltic Sea (control salinity 15). *M. arenaria* showed large individual variability in the burrowing efficiency, with an average of ~7% of the body energy reserves used per burial. Clams with higher mitochondrial capacity and lower energy expenditure per burial showed higher endurance. Acclimation for 3-4 weeks to low (5) or fluctuating (5-15) salinity reduced the burrowing speed and the number of times the clams can re-bury but did not affect the mitochondrial capacity of the whole body or the gill. Acclimation to the fluctuating salinity shifted the predominant fuel use for burrowing from proteins to lipids. Our data indicate that the reduced burrowing performance of clams under the salinity stress is not due to the limitations of energy availability or aerobic capacity but must involve other mechanisms (such as impaired muscle performance). The reduction in the burrowing capacity of clams due to salinity stress may have important implications for survival, activity and ecological functions of the clams in shallow coastal ecosystems.

ABBREVIATIONS

ADP	adenosine diphosphate
AM	assay media
ATP	adenosine triphosphate
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chlorophenyl hydrazine
COX	cytochrome c oxidase
E	energy expended for one digging cycle
E_s	total energy reserve (combustion energy equivalent of proteins, lipids, and carbohydrates)
E_c	total consumed energy (ETS capacity)
EGTA	ethylene bis (oxyethylenenitrilo) tetraacetic acid
ETS	electron transport system
FAAs	free amino acids
FS	fluctuating salinity (15-5)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HM	homogenization media
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride
LS	low salinity (5)
N_{ex}	number of times clams could dig until exhaustion
NADH	nicotinamide adenine dinucleotide (reduced form)
NS	normal salinity (15)
OXPHOS	oxidative phosphorylation
PL	proton leak
PMSF	phenylmethylsulfonyl fluoride
RCR	respiratory control ratio
rpm	rotations per minute
SUIT	substrate-uncoupler-inhibitor titration
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
ToD	time of disturbance (number of digging bouts)

Burrowing benthic organisms (bioturbators) serve as ecosystem engineers in soft-bottom marine ecosystems affecting biogeochemistry of marine sediments, pelagic-benthic coupling and secondary production (Meysman et al., 2006). Marine bivalves such as a soft-shell clam *Mya arenaria*, are key bioturbators in soft-bottom ecosystems around the world (Powers et al., 2006; Strasser, 1998) as well as important sentinel organisms in marine environmental monitoring (Borja et al., 2000; Lu, 2005). Benthic bivalves are commonly exposed to multiple stressors including fluctuations in salinity, temperature, oxygen content, pH, and pollutants in the sediment and overlaying water. Due to their sedentary lifestyle, bivalves have limited capacity to escape the unfavorable conditions and rely on physiological mechanisms to survive in the changing environment (Berger and Kharazova, 1997; Grieshaber et al., 1994; Sokolova et al., 2015; Sokolova et al., 2011). Physiological adjustments to environmental stress are energetically costly and may result in trade-offs between the maintenance and other fitness-related functions such as activity, growth, and reproduction (Sokolova, 2013; Sokolova et al., 2012). Understanding the impacts of multiple stressors on the bioenergetics of marine bioturbators is therefore important for predicting the potential effects of environmental shifts on survival and performance of their populations and on the ecological services they provide as ecosystem engineers.

Fluctuating salinity is an important stressor in shallow habitats including estuaries, coastal lagoons and semi-enclosed seas (McLusky and Elliott, 2004). Salinity regime can fluctuate due to the seasonal or short-term changes in precipitation, freshwater run-off and evaporation. Furthermore, alteration of the hydrological cycle due to the anthropogenic climate change results in the freshening of the surface sea waters and higher frequency of extreme precipitation events (Durack et al., 2012). In semi-enclosed brackish seas with extensive drainage areas such as the Baltic Sea this may result in major changes of the salinity regime due to the increased runoff (Vuorinen et al., 2015). Fluctuating salinity can affect marine bivalves by shifting the osmotic balance of their tissues and negatively impacting key cellular processes and the integrity of organelles and cell membranes (Berger and Kharazova, 1997; Prosser, 1991). A short-term survival of extreme salinities may be achieved by behavioral avoidance such as retracting deeper into the sediment or closing the shell to prevent contact with the ambient water; however, this behavior limits feeding and gas exchange and is not sustainable in the long-term (Sokolova et al., 2000a; Sokolova et al., 2000b; Truchot, 1993). Acclimation of marine bivalves to changing salinity involves regulation of the intracellular osmolarity to achieve balance with the external salinity and prevent swelling or shrinking of the cells (Berger and Kharazova, 1997; Pierce and Amende, 1981; Yancey, 2005). In osmoconformers such as mollusks, intracellular osmolarity is mainly maintained by controlling the amino acid concentrations (Davenport, 1985; Hawkins and Hilbish, 2009). As the ambient salinity increases, the intracellular pool of free amino acids (FAAs) increases due to the protein breakdown, and as salinity declines, the FAAs are catabolized or excreted thereby maintaining the intracellular fluid isosmotic to the environment (Berger and Kharazova, 1997; Pierce and Amende, 1981). The protein breakdown and subsequent loss of FAAs may incur considerable

energy costs to osmoconformers in fluctuating salinity environments (Hawkins and Hilbish, 2009) potentially impairing other energy demanding functions such as the burrowing activity.

Shallow coastal habitats experience high sediment dynamics due to the export from the land, storms, and the action of waves and tides (Carniello et al., 2014). Sediment reworking can expose bioturbators resulting in mortality due to predation or washing off up the shore, or bury them under additional sediment layers thereby preventing feeding and gas exchange (Emerson and Grant, 1991; Emerson et al., 1990; Powilleit et al., 2009; Probert, 1984). To survive these changes, bioturbators need to actively burrow to achieve the depth that allows them access to water and food yet provides protection against predators and/or abiotic extremes (Powilleit et al., 2009; Zwarts and Wanink, 1989). Burrowing is considered an energetically expensive mode of locomotion (Brown, 1979; Dorgan et al., 2011; Willmer et al., 2000). The direct estimates of the energy expense of burrowing are rare for marine organisms, but the available data indicate high energy costs of burrowing (Ansell and Trueman, 1973; Dorgan et al., 2011; Meysman et al., 2006; Trevor, 1978). Earlier studies showed that deviation of salinity from a species-specific optimum may impair burrowing behavior of sediment-dwelling organisms (Lardies et al., 2001; Przeslawski et al., 2009). However, the physiological mechanisms underlying the effects of salinity on the burrowing of marine bioturbators is not well understood, and the bioenergetic consequences of the combined salinity stress and repeated mechanical disturbance (such as may occur due to the wave action or sediment reworking) are not known, limiting our ability to make predictions about the impacts of these stressors on survival and ecological functions of bioturbators.

The aim of our study was to determine the combined effects of salinity stress and mechanical disturbance on the bioenergetics and burrowing behavior of the soft shell clam *M. arenaria*. *M. arenaria* is abundant in the shallow waters of the southwestern Baltic where it frequently experiences salinity fluctuations due to the exchange of water between the North and the Baltic Sea and due to the freshwater inputs from precipitation, land run-off and submarine groundwater discharge (Matthäus, 1995). *M. arenaria* can tolerate salinities from 4.5 to 35 and temperatures from -2°C to 28°C (Strasser, 1998) and is commonly exposed to the mechanical disturbance and dislodgement by the waves and tidal currents (Emerson and Grant, 1991; Redjah et al., 2010; St-Onge et al., 2007). We hypothesized that the combined effects of salinity stress and repeated burrowing will cause bioenergetic stress in *Mya arenaria,* and that elevated energy costs caused by the physiological adjustments to low or fluctuating salinity will impair burrowing activity of the clams. To test this hypothesis, we acclimated clams to different salinity regimes (normal – salinity 15, low – salinity 5 and fluctuating salinity regime involving daily salinity cycles between 15 and 5). Clams were subject to mechanical disturbance by exposing them on the surface, and their ability to re-bury and the average burial time was determined. We also assessed the effects of salinity, disturbance and their combination on tissue energy reserves (proteins, lipids and carbohydrates) and aerobic capacity of the clams. Our study provides insights into the physiological mechanisms and energy costs of the combined effects of salinity stress and mechanical disturbance on burrowing of *Mya arenaria* and has

implications for understanding the potential effects of these stressors on performance of a keystone marine bioturbator in shallow coastal habitats.

MATERIALS AND METHODS

Animal collection and maintenance. Soft shell clams (*Mya arenaria,* 10-70 mm shell length) were collected from Schnatermann, a shallow water area near Rostock, Germany, and transported in sea water to the University of Rostock. The clams were placed in a washed sandy sediment in recirculating aquarium systems at 15±0.5°C and salinity 15±1 (practical salinity units) for two weeks. This was close to the typical salinity at the study site (12 at the time of collection, 10-20 throughout the year). A randomly selected group of clams was gradually acclimated to low salinity (5) by reducing salinity at the rate of 1 per day until the target salinity of 5 was reached. Clams were then acclimated for 3-4 weeks at one of the following salinity regimes: 1) constant normal salinity (NS) 15; 2) constant low salinity (LS) 5, and 3) fluctuating salinity (FS) with the daily salinity changes between 5 and 15. In the FS treatment, the square salinity wave was applied as follows: 23 h at salinity 15, 1 h at salinity 10, 23 h at salinity 5, and 1 h at salinity 10, repeated for 3-4 weeks. Clams were fed *ad libitum* with DTs Premium Blend Live Phytoplankton (Coralsands, Wiesbaden, Germany) every other day (4 ml per 60-70 g of clam biomass).

Burial time. To assess the burrowing performance and exercise endurance of the clams, *M. arenaria* were exposed to repeated mechanical disturbance (every 45 min) and their burial time (i.e. the time required to escape from the sediment surface) and the number of times the clams could re-bury before becoming exhausted was determined. The behavioral trials were conducted at 15°C and salinity 15 (for clams acclimated at the normal and fluctuating salinity) or salinity 5 (for clams acclimated at low salinity). Clams (shell length 26.9±0.6 mm, N=67) were placed in shallow tanks filled with the washed sandy sediment and allowed to bury for 45 min. The behavior was recorded every 30 seconds, and the burial time was determined as the time that elapsed from the clam's dilation of the foot (indicating the start of digging) to the time the clam disappeared under the sediment surface (Fig. 1A). After 45 min the clams that dug into the sediment were gently pulled up, exposed, and left to bury for another 45 min. This cycle was repeated for 4-5 times (exercised group) or until the clams were unable to bury within the 45 min interval (exhausted group). Notably, once the clams were under the sediment surface, their behavior cannot be observed. However, the depth from which the clams were retrieved after the 45 min burial cycle (~3-7 cm) indicate that they continued digging once in the sediment. The preferred burial depth of the clams of the studied size range (10-14 cm) (Emerson et al., 1990; Zwarts and Wanink, 1989) has not been achieved during the 45 min experimental digging intervals. Control clams were maintained under the same salinity regimes but without disturbance. After the exposures, the control, exercised and exhausted clams were dissected, shock frozen in the liquid nitrogen and stored at -80°C.

Mitochondrial assays. Mitochondria were isolated from the gills of *M. arenaria* using differential centrifugation (Ivanina et al., 2016; Sokolova, 2004). The gill is the main respiratory organ of *M. arenaria*; therefore, the energetic performance of the gills is important for the overall aerobic performance of a burrowing clam. Clams acclimated to the FS regime were collected during the salinity 15 phase of the fluctuating salinity cycle, and clams acclimated to the NS or LS regime were collected at their respective acclimation salinities. Briefly, gills from two clams (0.6 – 0.9 g) were pooled and homogenized in 5 ml of mitochondrial homogenization media (HM). Gill mitochondria of clams acclimated to the NS and FS regimes were isolated using high osmolarity HM containing 100 mmol l^{-1} sucrose, 200 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl, 30 mmol l^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5), 8 mmol l^{-1} ethylene-bis-(oxyethylenenitrilo) tetraacetic acid (EGTA). Gill mitochondria from clams acclimated to the LS regime were isolated using low osmolarity HM containing 50 mmol l^{-1} sucrose, 100 mmol l^{-1} KCl, 50 mmol l^{-1} NaCl, 30 mmol l^{-1} HEPES (pH 7.5), 8 mmol l^{-1} EGTA. All HM were supplemented with protease inhibitors (1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and 2 µg ml^{-1} aprotinin). Adjustment of the osmolarity of the HM to the acclimation osmolarity was needed to isolate high quality mitochondria as assessed by the respiratory control ratio (Table 1) and mitochondrial integrity test using cytochrome c addition. Gill tissues were homogenized in ice-cold HM using several passes of a Potter–Elvenhjem homogenizer at 200 rpm. The homogenate was centrifuged at 4°C and 2,000 g for 4 min to remove cell debris, and the supernatant was centrifuged at 4°C and 8,500 g for 8 min to obtain a mitochondrial pellet. The mitochondria were washed once in the corresponding HM, collected by brief centrifugation (8,500 g for 5 min) and resuspended in ice-cold storage media containing 140 mmol l^{-1} taurine, 130 mmol l^{-1} KCl, 10 mmol l^{-1} NaCl, 30 mmol l^{-1} HEPES, 10 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl_2$ and 1% of fatty acid free bovine serum albumin (BSA). Mitochondrial functions were assessed using high resolution respirometry in one of the two assay media: 1) high osmolarity AM (450 mOsm, corresponding to salinity 15) - 140 mmol l^{-1} sucrose, 130 mmol l^{-1} KCl, 10 mmol l^{-1} NaCl, 30 mmol l^{-1} HEPES, 10 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl_2$, pH 7.2, and 2) low osmolarity AM (150 mOsm, corresponding to salinity 5) - 60 mmol l^{-1} KCl, 30 mmol l^{-1} HEPES, 10 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl_2$, pH 7.2.

Oxygen consumption of isolated mitochondria was measured using Oxygraph 2k high resolution respirometer (Oroboros, Innsbruck, Austria) at 15°C. Two-point calibration for 0% and 100% air saturation was conducted. The following substrate-uncoupler-inhibitor titration (SUIT) protocol was used: 5 mmol l^{-1} pyruvate with 2 mmol l^{-1} malate to stimulate Complex I (NADH-linked) respiration; 10 mmol l^{-1} succinate to stimulate the electron flow through Complex II; 2.5 mmol l^{-1} ADP to achieve state 3 (ADP-stimulated) respiration; 2.5 µmol l^{-1} oligomycin to inhibit mitochondrial F_o, F_1-ATPase and achieve state 4 respiration indicative of the proton leak; 7.5 mmol l^{-1} carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to uncouple the mitochondrial ETS; 1 µmol l^{-1} rotenone to inhibit electron flux through Complex I; 2.5 µmol l^{-1} antimycin A to

inhibit electron flux through Complex III; 0.5 mmol l^{-1}N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and 2 mmol l^{-1}ascorbate to stimulate activity of Complex IV (cytochrome c oxidase, or COX), and 20 mmol l^{-1} KCN to inhibit COX. Mitochondrial respiratory states and control indices (Table 1) were calculated as described elsewhere (Estabrook, 1967; Gnaiger, 2012). All mitochondrial assays were completed within 60 min after the isolation. Protein concentrations were measured in mitochondrial suspensions using a Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA, USA), and corrected for the BSA content of the assay media. Mitochondrial respiration rates were expressed as μmol O_2 min^{-1} g^{-1} protein.

Determination of energy reserves. Energy reserves and ETS capacity were assessed in the foot and in the rest of the body of *M. arenaria*. The foot mass represents <1% of the total body mass of the clam (the average wet masses of the foot and the body were 10.0±0.8 mg and 1.13±0.09 g, respectively, N=44-69), and therefore, the foot removal has negligible effect on the estimate of the total energy reserve of the body. Lipid, carbohydrate and protein concentrations were measured using colorimetric methods. Lipid content was measured using chloroform-methanol method (Van Handel, 1985). Approximately 50 mg of tissue powder (ground under the liquid nitrogen) was added to 3 ml of chloroform: methanol mixture (1:1, v: v) and incubated for 5 min with periodic vigorous mixing. The mixture was centrifuged at 3000 x *g* for 4 min at room temperature. 250 μl of samples or standards (soybean oil in acetone) were transferred to culture tubes and heated to 100°C for ~10-12 min until the solvent was evaporated. 100 μl of 98% sulfuric acid (H_2SO_4) was added to the dry sample, mixed and heated at 100°C for 10 min. The sample was cooled down to room temperature, mixed with 2.4 ml of vanillin reagent (600 mg vanillin in 100 ml of hot water and 400 ml of 85% phosphoric acid) and incubated for 5 min. The absorbance was measured at 490 nm using a SpectraMax M2 microplate reader (Molecular Devices GmbH, Biberach-an-der-Riß, Germany).

For determination of the carbohydrate and protein content, ~50 mg of tissue powder was mixed with ultrapure water containing 0.1% Triton (1:10 tissue mass to volume). Cells were lysed by three rapid freeze-thaw cycles of 5 min at -80°C followed by 5 min in 37°C water bath and centrifuged at 3000 x *g* for 3 min at room temperature. The supernatant was used for carbohydrate and protein measurements. Carbohydrate concentrations were measured using phenol-sulfuric acid method (Masuko et al., 2005). Samples or glucose standards (200 μl) were mixed with 600 μl of concentrated H_2SO_4, immediately followed by addition of 120 μl of 5% phenol. Samples were incubated for 5 min at 90°C, cooled and absorbance measured at 492 nm to calculate the carbohydrate content (in glucose equivalents). Protein concentrations were measured at 595 nm using Biorad Bradford Protein Assay Kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA) with BSA as a standard. The standard curves for lipids, carbohydrates and proteins showed good linearity (R^2=0.99). Total energy reserve was calculated by transforming the measured protein, lipid and carbohydrate into energy equivalent using their respective energy of combustion: 24 kJ g^{-1} for proteins, 39.5 kJ g^{-1} for lipids and 17.5 kJ g^{-1} for carbohydrates (Gnaiger, 1983).

Whole-body ETS capacity. ETS activity was measured using (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) (INT) tetrazolium reduction assay as described elsewhere (De Coen and Janssen, 1997; Owens and King, 1975). Whole body of clams was ground under the liquid nitrogen and homogenized with ETS-B solution (153 µM $MgSO_4$, 1.5 mg ml^{-1}polyvinylpyrrolidone, and 0.2% (v:v) Triton X-100 in 0.1 M Tris-HCl, pH 8.5) at 1:50 (w:v) ratio. The homogenate was centrifuged at 3000 x *g* for 10 min at 4°C, and supernatant used to assess the ETS activity as the rate of reduction of INT-tetrazolium to formazan at room temperature (22.5°C). Briefly, 28.5 µl of tissue homogenate was added to 85.5 µl of the buffer (0.13 M Tris-HCl and 0.3% (w/v) Triton X-100; pH 8.5) and 29 µl of NAD(P)H solution (1.7 mM NADH and 250 µM NADPH). 57 µl of 8 mM INT-tetrazolium was added to start the reaction and the absorbance was measured at 490 nm for 15 min at 2 min intervals. To account for potential non-mitochondrial reduction of INT-tetrazolium, blanks were ran for each sample by substituting 2 µl of 1 mM rotenone (an inhibitor of the mitochondrial Complex I) and 27 µM of 5 M KCN (a COX inhibitor) for the NAD(P)H solution. ETS activity was calculated by subtracting the blank slopes from the reaction slopes for each sample. Specific ETS activity of the tissues (µmol O_2 min^{-1} mg wet tissue mass) was calculated the extinction coefficient of formazan of 15.9 mM^{-1} cm^{-1} and corrected for the light path (0.729 cm). The potential energy expenditure (E_c) was calculated based on the ETS flux assuming the stoichiometric equivalent of 1 µmole formazan to 0.5 µmole O_2 and using oxyenthalpic equivalents for combustion of an average lipid, glycogen and protein mixture (484 J $mmole^{-1}$ O_2) (Gnaiger, 1983; Verslycke et al., 2004).

Statistics. Effects of salinity regime and exercise on bioenergetics traits of *M. arenaria* were tested using ANCOVA with salinity regime (NS, LS and FS) and exercise groups (control, exercised and exhausted) as fixed factors and size of the clams and the amount of times it has re-buried prior to sampling as covariates. Effects of acclimation salinity and assay osmolarity on the mitochondrial functions was tested using generalized linear model ANOVA with salinity regime (NS, FS and LS) and assay buffer osmolarity (150 and 450 mOsm) as fixed variables. The assumptions of normal distribution and homogeneity of variances were tested for all dependent variables using Shapiro-Wilk and Levine tests, respectively. Statistical outliers (0-2 per group) were detected using stem-and-leaf plot and removed from subsequent analysis. Following the outliers' removal, all data were normally distributed and the variances were homogeneous ($P>0.05$). The means for the burial time, tissue levels of energy reserves and ETS activity are corrected to the average common size of clams (26-27 mm) and the average number of times the clams were disturbed (n=4) based on the ANCOVA analyses.

To determine the effects of the salinity regime, energy costs of digging and clam size on the number of times the clam can re-bury during the repeated disturbance, a multiple regression analysis was used. In the initial model, clam size (shell length, L), salinity regime (S, encoded 1, 2 and 3 for NS, LS and FS, respectively) and the amount of energy expended during a single dig (E) were used as explanatory variables. The energy cost of digging (E) was calculated as a difference between the amount of energy reserves remaining in the clams' body after repeated

digging and the average amount of energy reserves in the undisturbed clams from the same salinity regime group, divided by the number of the digging bouts. Because the amount of energy reserves could not be measured non-invasively, average E_s of the undisturbed clams from the same salinity regime group was used as a proxy for the starting amount of energy reserves available to the clam. Cook's distance test was used to detect potential outliers; two significant outliers were detected and removed from the NS-acclimated group. Autocorrelation of the data was tested using Durbin-Watson test; no autocorrelation was detected. Because the effect of the clam size (L) on the energy cost of digging (E) was not significant (P=0.866), it was excluded from the final analysis. Due to the significant effects of the salinity regime on the number of times the clams were able to rebury, linear regressions describing the relationship between the energy costs of digging and the number of times the clams can rebury were constructed separately for each salinity group, and tested for significant differences in the intercepts and slopes.

Statistical analyses were conducted using IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism ver 7.02 (GraphPad Software Inc., La Jolla, CA, USA) software.

RESULTS

Time to bury. The average time required to bury after the disturbance differed between the clams of different sizes (R^2=0.693, P>0.05). Generally, larger clams were slower, with clams >40 mm shell length requiring over 1-2 h to bury (Fig. 1B). The relationship between the clam size and the time required to bury was non-linear, and the speed or burial was similar for clams of ~15-40 mm shell length (Fig. 1B). Therefore, only clams in the size range of ~20-40 mm were used in the behavioral and bioenergetic studies.

Clams acclimated at the low (5) or fluctuating (5 – 15) salinity took longer time to bury than the clams acclimated at the normal salinity (15) (Fig. 2A). There was no significant difference in the burial time between exercising and exhausted group at the normal and low salinity. At the fluctuating salinity, the average burial time was significantly longer in the clams that were allowed to re-bury until exhaustion compared to the exercised group collected after 3-4 cycles of digging (Fig. 2A). Acclimation to the constant low salinity (5) also had significant effect on the number of reburial before their exhaustion (Fig. 2B). In the constant low salinity, clams could rebury 4-5 times whereas in the normal salinity they can rebury up to 9-10 times before exhaustion. A similar (albeit statistically non-significant) trend for lower endurance (i.e. fewer number of times that clams can re-bury) was found in the clams acclimated to the fluctuating salinity compared to the NS clams (Fig. 2B).

Mitochondrial functions in the gill. Acclimation to different salinity regimes affected the ability of the gill mitochondria of the clams to perform at different osmolarities (Fig. 3). In clams acclimated to normal salinity (15), the oxidation phosphorylation (OXPHOS) capacity and cytochrome oxidase (COX) activity of gill mitochondria were suppressed when measured in the low osmolarity media (150 mOsm) compared to the high osmolarity media (450 mOsm) (Fig.

3A, D). Proton leak rates were not affected by the assay osmolarity in gill mitochondria of NS clams (Fig. 3B). As a result, RCR of NS clams' mitochondria were lower at 150 mOsm indicating reduced mitochondrial efficiency compared to 450 mOsm (Fig. 3C). In contrast, in clams acclimated to LS or FS, gill mitochondria performed equally well at 150 and 450 mOsm (Fig. 3). When compared at 450 mOsm, key mitochondrial functions including the OXPHOS capacity, proton leak, COX activity and RCR were not affected by the salinity regime at which the clams were acclimated (Fig. 3). At 150 mOsm, the gill mitochondria of LS and FS clams generally outperformed those of NS clams (Fig. 3).

The apparent reserve capacity of ETS (the ratio of the ETS capacity to the OXPHOS rates) was relatively small (20-38%) in clam gill mitochondria (Fig. 3E) indicating that ETS activity is a good estimate of the ATP synthesis capacity in this species. Similarly, the apparent reserve of the mitochondrial COX capacity (the ratio of COX to ETS flux) was 16-42% (Fig. 3F). ETS/OXPHOS ratio was higher and COX/ETS ratio lower in the mitochondria of clams acclimated at LS and measured at 150 mOsm, reflecting the relatively high ETS flux in this group compared to their counterparts from the NS and FS regimes.

Energy reserves. The energy content per gram tissue was similar the foot muscle and the rest of the clam body (2.54±0.13 kJ g^{-1}, N=26 vs. 2.00±0.07 kJ g^{-1}, N=18, respectively). In the foot, ~35-40% of the total energy was contained in the proteins, ~50-60% contained in the lipids, and 4-5% - in carbohydrates. In the rest of the body, the contributions of proteins, lipids and carbohydrates to the total energy reserves were ~60%, 30-35% and 6-8%, respectively. Acclimation to the constant low salinity (5) led to a slight but significant increase in the content of the proteins and carbohydrates in the foot of the clams, while acclimation to the fluctuating salinity had no effect on these traits (Fig. 4). Lipid content in the foot was not affected by acclimation to the fluctuating salinity (no data are available for the low salinity due to the sample loss) (Fig. 4). Due to the small size of the foot, the energy content of the foot was <0.2% of the total body energy reserves. No depletion of energy reserves was found in the foot of digging clams (data not shown).

Repeated digging resulted in a significant decline of the energy reserves of the total body of the clams regardless of the acclimation salinity (Fig. 5A). Notably, clams acclimated to different salinity regimes depended on different types of energy reserves to fuel digging activity. The body protein reserves were significantly lower (by ~20-30%) in the undisturbed clams acclimated to the fluctuating salinity regime compared to their counterparts acclimated to the normal or constantly low salinity (Fig. 5B). During repeated digging, the body levels of the proteins declined in the clams acclimated to the normal or constantly low salinity, but not in those acclimated to the fluctuating salinity (Fig. 5B). In contrast, the lipid reserves were higher (by ~30-50%) in the undisturbed clams acclimated to the fluctuating salinity compared to those from the normal and low salinity regimes (Fig. 5C). The lipid content decreased after repeated digging in the exercising clams at all three studied salinity regimes, and in the exhausted clams in the fluctuating salinity (Fig. 5C). Carbohydrate reserves were ~20-25% higher in the clams

acclimated to the constant low salinity compared to their counterparts from the normal or fluctuating salinity regimes (Fig. 5D). Carbohydrate reserves were significantly decreased after the exhausting exercise in the clams at the normal and constantly low salinity, but not in those acclimated to the fluctuating salinity (Fig. 5D). Overall, the clams acclimated to the normal or constantly low salinity covered ~68-69% of the energy requirements for digging from the protein reserves, ~26-27% from the lipids and ~3-5% from the carbohydrate reserves. In contrast, clams acclimated to the fluctuating salinity covered ~27% of the energy cost of digging from the protein reserves, ~71% from the lipids and ~2% from the carbohydrates.

Aerobic ETS capacity. Acclimation salinity has no significant effect on the total ETS activity in clam tissues (Fig. 6A). Clams acclimated to fluctuating salinity showed an increase in the tissue ETS capacity following exercise, which was especially pronounced in the exhausted group (Fig. 6A). Repeated digging had no effect on the total body ETS capacity in the clams acclimated to NS or LS regimes. The ratio of energy reserves to the potential energy expenditure (E_s/E_c) was not affected by the salinity regime but declined after the repeated exercise in NS, LS and FS clams (Fig. 6B).

Energy cost of repeated digging. The number of times a clam could repeatedly dig until exhausted was negatively correlated with the energy cost of reburial (expressed in J g^{-1} wet mass per burial) (Fig. 7A). Depending on the acclimation salinity, 69-77% of the variation in the number of re-burials until exhaustion was explained by the variation in the energy expenditure per dig. The multiple regression analysis showed that both the salinity regime (S) and the amount of energy expended during a single dig (E) significantly affected the number of times the clams could dig until exhausted (N_{ex}): N_{ex}=11.22-1.21xS-0.02xE (adjusted R^2=0.739; $P<0.01$ for the constant, S and E). The relationship between the energy cost of an individual dig and the number of times the clam could re-bury was independent of the clam size in the studied size range ($P>0.05$ for the effect of size in the multiple regression analysis). The regressions of the number of times the clams could re-bury on the energy expenditure per burial for clams acclimated to the different salinity regimes had the same slopes ($P>0.05$) but different intercepts ($P<0.05$) (Fig. 7A). This indicates that at the same energy expenditure per dig, clams acclimated at low and fluctuating salinity could re-bury fewer times before becoming exhausted than their normal-salinity counterparts.

The energy expenditure per dig was negatively correlated with the ETS capacity of the clams, so that the clams that have low ETS capacity spent relatively more energy per dig (Fig. 7B). The regression was significant ($P<0.01$), but the variation in the ETS capacity explained only ~20% of the variation in the energy costs of digging.

DISCUSSION

Acclimation salinity, body size and bioenergetics efficiency of digging behavior are the important determinants of the burrowing capacity of a common marine bioturbator *M. arenaria*. The burrowing performance of clams was negatively affected by low and fluctuating salinity (such as commonly occurs in estuaries and shallow coastal habitats) and positively correlated with the individual bioenergetic efficiency of digging. Regardless of the salinity regime, smaller *M. arenaria* were capable of burrowing faster after the disturbance compared to their larger counterparts. These findings agree with the results of earlier studies showing that smaller clams dig faster (Emerson *et al.*, 1990; Checa and Cadeé, 1997) and more frequently (St-Onge *et al.*, 2007) than the larger ones. This may reflect biomechanical constraints of digging due to the relatively smaller foot size in the large clams and/or the possible bioenergetics constraints due to the greater resistance of the sediments for propelling a larger body (Checa and Cadée, 1997; Dorgan, 2015; St-Onge et al., 2007). It is worth noting, however, that large clams are likely to be less exposed to the disturbance due to the wave action because of their greater burial depth so that they only become exposed during the strongest storms (Redjah et al., 2010; Zaklan and Ydenberg, 1997; Zwarts and Wanink, 1989). Therefore, frequent disturbance is the most relevant stressor for the smaller clams such as used in the present study that typically inhabit the upper 10-15 cm of the sediment (Emerson and Grant, 1991; Emerson et al., 1990; Redjah et al., 2010; Zwarts and Wanink, 1989).

Energy costs of M. arenaria digging

In all studied salinity regimes, *M. arenaria* started digging after some delay after exposure (~7-90 minutes, depending on the clam size), in agreement with the earlier findings (Breum, 1970). Once the digging commenced, it proceeded rapidly, and the clams were fully buried within 20-32 min, depending on the clam size. Indirect estimates of energy expense of digging (based on the depletion of energy reserves) show that one digging cycle of *M. arenaria* (with an average duration of 25-35 min) requires 0.109 ±0.02 J mg^{-1} body mass corresponding to 6 x 10^{-5} J mg^{-1} s^{-1}. These values are comparable to those reported for other burrowing bivalves (5 x 10^{-4} J s^{-1} and 3.6 x 10^{-4} J s^{-1} for *Donax denticulatus* and *Donax incarnates*, respectively) (Ansell and Trueman, 1973) and higher than in gastropods (1.48 x 10^{-8} J mg^{-1} s^{-1} for *Bullia digitalis*) (Brown, 1979) and polychaetes (4.8 x 10^{-10} and 3.3 x 10^{-9} J mg^{-1} s^{-1} for *Cirriformia moorei* – digging in gelatin and sediment, 104.3 x 10^{-7}, 3.1 x 10^{-7}, and 2.8 x 10^{-7} J mg^{-1} s^{-1} for *Nephtys cirrosa*, *Nereis diversicolor* and *Arenicola marina*, respectively, digging in sediment) (Dorgan et al., 2011; Trevor, 1978). The daily energy requirement for burial of a surf *Donax incarnatus* (which repeatedly buries to maintain its vertical position in the sand and to move along the shore during the tidal cycle) was estimated at >30% of the routine metabolism (Ansell and Trueman, 1973). The respective estimate of the daily energy budget for digging is not available for *M. arenaria*; however, our data indicate that even a single burial cycle incurs a considerable energy cost using up on average 7% of the total body energy reserves of *M. arenaria*. Interestingly, the

bioenergetic estimates of burrowing terrestrial species (such as the rodents) also indicate high energy costs of the burrowing activity despite the differences in the environmental conditions as well as the burrowing physiology and biomechanics compared to aquatic bioturbators (Lovegrove, 1989; Vleck, 1979). Thus, the oxygen consumption rates of burrowing rodents *Thomomys bottae*, *Cryptomys damarensis* and *Heterocephalus glaber* are 4-5 times higher during digging compared to the routine metabolism (Lovegrove, 1989; Vleck, 1979) indicating high energy costs of burrowing. Taken together, these data support the notion that digging is an energetically expensive activity in aquatic as well as terrestrial bioturbators.

High energy costs of burrowing imply that the ability to re-bury after repeated disturbance may become limited when the energy reserves are exhausted below a certain threshold level. Our data in *M. arenaria* support this hypothesis showing that when the body energy reserves dropped by 20-27%, clams were no longer able to re-bury. Notably, individual variability in the energetic efficiency of digging was associated with the ability to sustain repeated digging activity in *M. arenaria*, so that the clams that spent less energy per unit body mass in an individual digging bout were able to dig more times before becoming exhausted. This indicates that the more energy-efficient diggers may have greater chances of survival in shallow habitats exposed to wind and wave action, and supports the notion that sustained digging activity may be energetically limited. Depletion of the whole-body energy reserves during digging shows that repeated burrowing activity has global bioenergetic consequences for *M. arenaria* and may result in trade-offs with other energy demanding fitness-related functions such as growth, reproduction, immune defense or basal maintenance.

Sustained digging of marine bioturbators including such clams requires high aerobic capacity to support the elevated ATP demand (Morley et al., 2007), and stressors that diminish the organism's aerobic scope (such as extreme temperatures or decreasing oxygen availability) may negatively impact the burrowing capacity of clams (as shown in an Antarctic clam *Laternula elliptica)* (Peck et al., 2007). Our study shows that the whole-body aerobic capacity (measured as ETS activity) is maintained at high levels (or even increases as observed in the FS clams exercised until exhaustion) in *M. arenaria* subjected to repeated exercise. Although the ETS capacity was measured in the whole body rather than the exercising muscle, high aerobic capacity might assist in the global mobilization of the energy reserves to support repeated digging. Notably, high ETS capacity was associated with higher energy efficiency (i.e. lower energy expenditure per unit mass) of digging in *M. arenaria* (Fig. 6B). The mechanisms underlying this correlation are not known; however, our data indicate that both high capacity of aerobic metabolism and energetic efficiency of exercise may be important factors for *M. arenaria* survival in wind-disturbed shallow habitats.

Effects of salinity regime on bioenergetics of M. arenaria burrowing

Acclimation at low (5) or fluctuating (5-15) salinity increased the average amount of time needed by the clams to re-bury after a disturbance by 7-15%. The clams were also rapidly exhausted (after 5-6 digging cycles) in LS and FS regimes compared to their counterparts

maintained at the normal salinity who became exhausted after ~9 digging cycles. This indicates that salinity stress impairs the burrowing speed and stamina of *M. arenaria*. Similarly, hypoosmotic stress (salinity 17) increased the burial time of a brittle star, *Ophiophragmus filograneus* compared to the normal salinity (21) (Turner and Meyer, 1980). A decrease in salinity from 35 to 22 suppressed the burrowing activity of a polychaete *Capitella sp*. (measured as the sediment area covered by burrows), albeit this effect was partially reversed at salinity 16 (Przeslawski et al., 2009). Low salinity also negatively impacted the burial depth of tropical infaunal bivalves *Venus antiqua* and *Tagelus dombeii* leading to shallower burrowing in winter when the salinity was low (24) compared to the summer when the prevailing salinity was 32.5 (Lardies et al., 2001). Taken together, these data indicate that hyposalinity stress may negatively affect burrowing activity of marine bioturbators decreasing the biological reworking of the sediment and increasing the risks of predation and displacement by waves.

The negative impacts of the low and fluctuating salinity on burrowing activity of *M. arenaria* cannot be explained by the energy deficit induced by salinity stress. The total amount of energy reserves in clams' bodies was not affected by the acclimation salinity, and the relative amount of energy (20-27%) spent before the clams became exhausted and lost the ability to re-bury was similar in all groups regardless of the salinity regime. Furthermore, the relationship between the energy efficiency of digging and the number of times the clams can re-bury before exhaustion was similar in the clams acclimated to the normal, low and fluctuating salinity, despite the generally lower number of times the LS and FS clams could re-bury. This indicates that the burrowing performance of the LS and FS clams is impaired despite the similar energy demand of burrowing at the different salinities and suggests a direct negative effect of the low and fluctuating salinity of the contractile apparatus of the clams' muscles. The mechanisms of such salinity-induced impairment of the muscle properties are presently unknown. It is unlikely that this impairment involves a loss of the aerobic capacity of *M. arenaria* tissues. The whole-body ETS capacity was similar among all salinity acclimation groups of *M. arenaria*, and although acute hypoosmotic stress suppressed COX activity and reduced the coupling efficiency in gill mitochondria of *M. arenaria*, these effects were reversed and the normal mitochondrial function restored after the prolonged acclimation in the LS or FS regimes.

It is worth noting that the mitochondrial capacity was measured in the gill, which is the main organ for oxygen uptake. It is possible that low and fluctuating salinity have tissue-specific negative effects on the muscle mitochondria different from those in the gills and other tissues; however, this explanation appears unlikely since the mitochondrial responses to osmolarity change are similar in different tissues of marine bivalves (Ballantyne and Moon, 1985; Ballantyne and Moyes, 1987a; Ballantyne and Moyes, 1987b; Ballantyne and Moyes, 1987c; Ballantyne and Storey, 1983; Ballantyne and Storey, 1984). Another possibility is the loss of the contractile proteins in *M. arenaria* in response to salinity stress. This mechanism may partially explain the suppression of the burrowing activity in *M. arenaria* from the FS but not from the LS regime. Fluctuating salinity is known to result in the protein breakdown in marine osmoconformers such as marine mollusks (Berger and Kharazova, 1997; Hilbish et al., 1982;

Pierce and Amende, 1981). During the salinity upshift, cellular proteins are broken down to increase the cytosol osmolarity by accumulation of the free amino acids (FAAs) and thereby prevent cell shrinkage. When salinity decreases, the FAAs are metabolized and/or excreted to rapidly lower intracellular osmolarity. The repeated cycles of the up- and down-shifts of salinity thus result in the net protein loss, which constitutes a significant fitness cost of isosmotic cell volume regulation in marine osmoconformers (Hawkins and Hilbish, 2009; Hilbish et al., 1982). Constant salinity (even if it is suboptimal) does not have the same effects on the protein breakdown, and once the osmotic equilibrium with the ambient water is re-established, protein breakdown rates return to the steady state (Hawkins and Hilbish, 2009). In agreement with this mechanism, tissue protein content of *M. arenaria* clams acclimated in the fluctuating salinity regime was significantly lower than in the clams acclimated to the constant normal or low salinity.

A decrease in the amount of the protein reserves in the body caused by the acclimation to fluctuating salinity regime in *M. arenaria* was compensated by an increase in the lipid content so that the total amount of the energetic reserves of the body did not change. While the mechanisms responsible for the protein breakdown to maintain intracellular osmolarity in marine bivalves are well understood (Deaton et al., 1984; Hawkins and Hilbish, 2009), salinity effects on the lipid metabolism are less known. The Eastern oysters *Crassostrea virginica* acclimated at a reduced salinity had higher levels of phospholipids than their counterparts acclimated under the normal salinity regime (Glémet and Ballantyne, 1995). However, in marine fish (such as Atlantic salmon and turbot) tissue lipid content increased with increasing salinity (Tocher et al., 1994), whereas in a euryhaline guppy *Poecilia reticulata* transition from the fresh to sea water led to an decrease of the total body lipid content even though the lipid content increased locally in osmoregulatory organs (gills, digestive tract and kidney) (Daikoku et al., 1982). Although the underlying molecular mechanisms remain unknown, lipid accumulation in *M. arenaria* acclimated to the fluctuating salinity has implications for the bioenergetics of digging. While the energy cost of digging of clams acclimated to the constant salinity 5 or 15 was predominantly supported by the breakdown of proteins, digging activity of the clams acclimated to the fluctuating salinity was mostly fueled by catabolism of the lipids. Contribution of carbohydrate breakdown to covering the energy demand of digging was minimal in all experimental groups of *M. arenaria* irrespective of the acclimation salinity. This was an unexpected finding because in other animals (including birds, mammals, fish as well as the active mollusks such as scallops that can swim to escape predators), vigorous aerobic exercise is predominantly fueled by carbohydrates such as glycogen (Sahlin, 2009; Spriet, 2014). However, the fuel preference for aerobic exercise depends on the metabolic environment and the relative levels of different types of substrates in the working muscle (Spriet, 2014), so that higher dependence on the lipid catabolism in the digging FS clams may reflect higher availability of this fuel type compared to proteins or glycogen. Furthermore, while energetically expensive, digging of *M. arenaria* is a relatively slow locomotion, so that the force generated by the contracting muscle is likely to be more important for propelling the body than the speed of the muscle contractions. This may explain the greater reliance of digging *M. arenaria* on “slow”

metabolic fuels such as proteins or lipids when compared to the fast aerobic locomotion such as swimming or running where carbohydrates play an important role (Spriet, 2014; Willmer et al., 2000).

Significance and perspectives

Long-term exposure to low or fluctuating salinity may affect the survival and ecological functions of the soft shell clam *M. arenaria* exposed to freshwater influx and wave-induced sediment reworking in shallow coastal habitats. Repeated burrowing following the wave-induced disturbance is energetically expensive and can cause trade-offs with other energy-dependent activities (such as growth or reproduction) as well as increase the risk of predation and/or wash-off thereby leading to the population declines. Suppression of the burrowing capacity and speed of clams in the low and/or fluctuating salinities may exacerbate the problem; however, the negative effects of salinity on the clam burrowing are unlikely to reflect bioenergetics limitations (such as lower availability of the energy reserves or suppressed mitochondrial capacity) and must involve other mechanisms (such as the negative impacts of salinity stress on the contractile properties of the muscles involved in the hydraulic burrowing) (Checa and Cadée, 1997; Dorgan, 2015). The reduction in the burrowing capacity of clams due to salinity stress and the resulting impacts on clam activity and survival may have important implications for bioturbation and the sediment biogeochemistry of the shallow coastal ecosystems which require further investigation.

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References

Ansell, A. D. and Trueman, E. R. (1973). The energy cost of migration of the bivalve Donax on tropical sandy beaches. *Marine Behaviour and Physiology* **2**, 21-32.

Ballantyne, J. S. and Moon, T. W. (1985). Hepatopancreas mitochondria from *Mytilus edulis*: substrate preferences and effects of pH and osmolarity. *Marine Biology* **87**, 239-244.

Ballantyne, J. S. and Moyes, C. D. (1987a). The effects of salinity acclimation on the osmotic properties of mitochondria from the gill of *Crassostrea virginica*. *Journal of Experimental Biology* **133**, 449-456.

Ballantyne, J. S. and Moyes, C. D. (1987b). Osmotic effects on fatty acid, pyruvate, and ketone body oxidation in oyster gill mitochondria. *Physiol. Zool.* **60**, 713-721.

Ballantyne, J. S. and Moyes, C. D. (1987c). The role of divalent cations and ionic strength in the osmotic sensitivity of glutamate oxidation in oyster gill mitochondria. *Journal of Experimental Biology* **130**, 203-217.

Ballantyne, J. S. and Storey, K. B. (1983). Mitochondria from the ventricle of the marine clam, *Mercenaria mercenaria*: substrate preferences and effects of pH and salt concentration on proline oxidation. *Comparative Biochemistry and Physiology, Part B* **76B**, 133-138.

Ballantyne, J. S. and Storey, K. B. (1984). Mitochondria from the hepatopancreas of the marine clam *Mercenaria mercenaria*: substrate preferences and salt and pH effects on the oxidation of palmitoyl-L-carnitine and succinate. *The Journal of Experimental Zoology* **230**, 165-174.

Berger, V. J. and Kharazova, A. D. (1997). Mechanisms of salinity adaptations in marine molluscs. *Hydrobiologia* **355**, 115-126.

Borja, A., Franco, J. and Pérez, V. (2000). A Marine Biotic Index to Establish the Ecological Quality of Soft-Bottom Benthos Within European Estuarine and Coastal Environments. *Marine Pollution Bulletin* **40**, 1100-1114.

Breum, O. (1970). Stimulation of burrowing activity by wave action in some marine bivalves. *Ophelia* **8**, 197-207.

Brown, A. C. (1979). The energy cost and efficiency of burrowing in the sandy-beach whelk Bullia digitalis (Dillwyn) (Nassariidae). *Journal of Experimental Marine Biology and Ecology* **40**, 149-154.

Carniello, L., Silvestri, S., Marani, M., D'Alpaos, A., Volpe, V. and Defina, A. (2014). Sediment dynamics in shallow tidal basins: In situ observations, satellite retrievals, and numerical modeling in the Venice Lagoon. *Journal of Geophysical Research: Earth Surface* **119**, 802-815.

Checa, A. G. and Cadée, G. C. (1997). Hydraulic burrowing in the bivalve *Mya arenaria* Linnaeus (Myoidea) and associated ligamental adaptations *Journal of Molluscan Studies* **63**, 157-171.

Daikoku, T., Yano, I. and Masul, M. (1982). Lipid and fatty acid compositions and their changes in the different organs and tissues of guppy, poecilia reticulata on sea water adaptation. *Comparative Biochemistry and Physiology Part A: Physiology* **73**, 167-174.

Davenport, J. (1985). Osmotic control in marine invertebrates. *In: Physiological adaptations of marine animals. Symposia of the Society for experimental biology. M.S.Laverack [Ed.] The Company of Biologists LTD, Cambridge, U.K.* **39**, 207-244.

De Coen, W. M. and Janssen, C. R. (1997). The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. *Journal of Aquatic Ecosystem Stress and Recovery* **6**, 43-55.

Deaton, L. E., Hilbish, T. J. and Koehn, R. K. (1984). Protein as a source of amino nitrogen during hyperosmotic volume regulation in the mussel *Mytilus edulis*. *Physiological Zoology* **57**, 609-619.

Dorgan, K. M. (2015). The biomechanics of burrowing and boring. *The Journal of Experimental Biology* **218**, 176-183.

Dorgan, K. M., Lefebvre, S., Stillman, J. H. and Koehl, M. A. R. (2011). Energetics of burrowing by the cirratulid polychaete Cirriformia moorei. *Journal of Experimental Biology* **214**, 2202-2214.

Durack, P. J., Wijffels, S. E. and Matear, R. J. (2012). Ocean Salinities Reveal Strong Global Water Cycle Intensification During 1950 to 2000. *Science* **336**, 455-458.

Emerson, C. W. and Grant, J. (1991). The control of soft-shell clam (*Mya arenaria*) recruitment on intertidal sandflats by bedload sediment transport. *Limnology and Oceanography* **36**, 1288-1300.

Emerson, C. W., Grant, J. and Rowell, T. W. (1990). Indirect effects of clam digging on the viability of soft-shell clams, Mya arenaria L. *Netherlands Journal of Sea Research* **27**, 109-118.

Estabrook, R. W. (1967). Mitochondrial respiratory control and the polarographic measurements in mitochondria. *Methods in Enzymology* **10**, 41-47.

Glémet, H. C. and Ballantyne, J. S. (1995). Influences of environmental salinity on the structure and function of gill mitochondrial membranes of an osmoconforming invertebrate, Crassostrea virginica. *Marine Biology* **121**, 673-683.

Gnaiger, E. (1983). Calculation of Energetic and Biochemical Equivalents of Respiratory Oxygen Consumption. In *Polarographic Oxygen Sensors: Aquatic and Physiological Applications*, eds. E. Gnaiger and H. Forstner), pp. 337-345. Berlin, Heidelberg: Springer Berlin Heidelberg.

Gnaiger, E. (2012). Mitochondrial pathways and respiratory control: An introduction to OXPHOS analysis. *Mitochondrial Physiological Network (MiPNet) 17.18, OROBOROS MiPNet Publications 2012. Innsbruck.*, 1-64. Open access: http://www.bioblast.at/index.php/Gnaiger_2012_MitoPathways.

Grieshaber, M. K., Hardewig, I., Kreutzer, U. and Pörtner, H. O. (1994). Physiological and metabolic responses to hypoxia in invertebrates. *Reviews of physiology, biochemistry and pharmacology* **125**, 43-147.

Hawkins, A. J. S. and Hilbish, T. J. (2009). The costs of cell volume regulation: protein metabolism during hyperosmotic adjustment. *Journal of the marine biological association of the united kingdom* **72**, 569-578.

Hilbish, T. J., Deaton, L. E. and Koehn, R. K. (1982). Effect of an allozyme polymorphism on regulation of cell volume. *Nature* **289**, 688-689.

Ivanina, A. I., Nesmelova, I., Leamy, L., Sokolov, E. P. and Sokolova, I. M. (2016). Intermittent hypoxia leads to functional reorganization of mitochondria and affects cellular bioenergetics in marine molluscs. *Journal of Experimental Biology* **In press**.

Lardies, M. A., Clasing, E., Navarro, J. M. and Stead, R. A. (2001). Effects of environmental variables on burial depth of two infaunal bivalves inhabiting a tidal flat in southern Chile. *Journal of the marine biological association of the united kingdom* **81**, 809-816.

Lovegrove, B. G. (1989). The Cost of Burrowing by the Social Mole Rats (Bathyergidae) Cryptomys damarensis and Heterocephalus glaber: The Role of Soil Moisture. *Physiological Zoology* **62**, 449-469.

Lu, L. (2005). The relationship between soft-bottom macrobenthic communities and environmental variables in Singaporean waters. *Marine Pollution Bulletin* **51**, 1034-1040.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I. and Lee, Y. C. (2005). Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry* **339**, 69-72.

Matthäus, W. (1995). Natural variability and human impacts reflected in longterm changes in the baltic deep water conditions—A brief review. *Deutsche Hydrografische Zeitschrift* **47**, 47-65.

McLusky, D. S. and Elliott, M. (2004). The Estuarine Ecosystem. Ecology, Threats and Management: Oxford University Press.

Meysman, F. J. R., Middelburg, J. J. and Heip, C. H. R. (2006). Bioturbation: a fresh look at Darwin's last idea. *TRENDS in Ecology & Evolution* **21**, 688-695.

Morley, S. A., Peck, L. S., Tan, K. S., Martin, S. M. and Pörtner, H. O. (2007). Slowest of the slow: latitudinal insensitivity of burrowing capacity in the bivalve Laternula. *Marine Biology* **151**, 1823-1830.

Owens, T. G. and King, F. D. (1975). The measurement of respiratory electron-transport-system activity in marine zooplankton. *Marine Biology* **30**, 27-36.

Peck, L. S., Morley, S. A., Pörtner, H.-O. and Clark, M. S. (2007). Thermal limits of burrowing capacity are linked to oxygen availability and size in the Antarctic clam Laternula elliptica. *Oecologia* **154**, 479-484.

Pierce, S. K. and Amende, L. M. (1981). Control mechanisms of amino acid-mediated cell volume regulation in salinity-stressed molluscs. *Journal of Experimental Zoology* **215**, 247-257.

Powers, S. P., Bishop, M. A., Grabowski, J. H. and Peterson, C. H. (2006). Distribution of the invasive bivalve Mya arenaria L. on intertidal flats of southcentral Alaska. *Journal of Sea Research* **55**, 207-216.

Powilleit, M., Graf, G., Kleine, J., Riethmüller, R., Stockmann, K., Wetzel, M. A. and Koop, J. H. E. (2009). Experiments on the survival of six brackish macro-invertebrates from the Baltic Sea after dredged spoil coverage and its implications for the field. *Journal of Marine Systems* **75**, 441-451.

Probert, P. K. (1984). Disturbance, sediment stability, and trophic structure of soft-bottom communities. *Journal of Marine Research* **42**, 893-921.

Prosser, C. L. (1991). Environmental and Metabolic Animal Physiology. New york etc.: Wiley-Liss.

Przeslawski, R., Zhu, Q. and Aller, R. (2009). Effects of abiotic stressors on infaunal burrowing and associated sediment characteristics. *Marine Ecology Progress Series* **392**, 33-42.

Redjah, I., Olivier, F., Tremblay, R., Myrand, B., Pernet, F., Neumeier, U. and Chevarie, L. (2010). The importance of turbulent kinetic energy on transport of juvenile clams (*Mya arenaria*). *Aquaculture* **307**, 20-28.

Sahlin, K. (2009). Control of lipid oxidation at the mitochondrial level. *Appl Physiol Nutr Metab* **34**, 382-8.

Sokolova, I. M. (2004). Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *Journal of Experimental Biology* **207**, 2639-2648.

Sokolova, I. M. (2013). Energy-Limited Tolerance to Stress as a Conceptual Framework to Integrate the Effects of Multiple Stressors. *Integrative and Comparative Biology* **53**, 597-608.

Sokolova, I. M., Bock, C. and Pörtner, H. O. (2000a). Resistance to freshwater exposure in White Sea *Littorina* spp. I: Anaerobic metabolism and energetics. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **170**, 91-103.

Sokolova, I. M., Bock, C. and Pörtner, H. O. (2000b). Resistance to freshwater exposure in White Sea *Littorina* spp. II: Acid-base regulation. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **170**, 105-115.

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research* **79**, 1-15.

Sokolova, I. M., Matoo, O., Dickinson, G. and Beniash, E. (2015). Physiological effects of ocean acidification on animal calcifiers. *In: Stressors in the Marine Environments. N. Whiteley & M. Solan (eds.) Oxford University Press, Oxford, U.K.*

Sokolova, I. M., Sukhotin, A. A. and Lannig, G. (2011). Stress effects on metabolism and energy budgets in mollusks *In: Oxidative Stress in Aquatic Ecosystems. D. Abele, T. Zenteno-Savin, J. Vazquez-Medina (eds). Blackwell Wiley, Boston etc.*, 263-280.

Spriet, L. L. (2014). New insights into the interaction of carbohydrate and fat metabolism during exercise. *Sports Med* **44 Suppl 1**, S87-96.

St-Onge, P., Miron, G. and Moreau, G. (2007). Burrowing behaviour of the softshell clam (*Mya arenaria*) following erosion and transport. *Journal of Experimental Marine Biology and Ecology* **340**, 103-111.

Strasser, M. (1998). Mya arenaria — an ancient invader of the North Sea coast. *Helgolander Meeresuntersuchungen* **52**, 309-324.

Tocher, D. R., Castell, J. D., Dick, J. R. and Sargent, J. R. (1994). Effects of salinity on the growth and lipid composition of Atlantic salmon (Salmo salar) and turbot (Scophthalmus maximus) cells in culture. *Fish Physiol Biochem* **13**, 451-61.

Trevor, J. H. (1978). The dynamics and mechanical energy expenditure of the polychaetes Nephtys cirrosa, Nereis diversicolor and Arenicola marina during burrowing. *Estuarine and Coastal Marine Science* **6**, 605-619.

Truchot, J. P. (1993). Acid-base balance in aquatic invertebrates: the effect of environmental factors. *Coastal and Estuarine Studies* **43**, 1-14.

Turner, R. L. and Meyer, C. E. (1980). Salinity Tolerance of the Brackish-Water Echinoderm *Ophiophragmus filograneus* (Ophiuroidea) *Marine Ecology Progress Series* **2**, 249-256.

Van Handel, E. (1985). Rapid determination of total lipids in mosquitoes. *J Am Mosq Control Assoc* **1**, 302-4.

Verslycke, T., Roast, S. D., Widdows, J., Jones, M. B. and Janssen, C. R. (2004). Cellular energy allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison. *Journal of Experimental Marine Biology and Ecology* **306**, 1-16.

Vleck, D. (1979). The Energy Cost of Burrowing by the Pocket Gopher Thomomys bottae. *Physiological Zoology* **52**, 122-136.

Vuorinen, I., Hänninen, J., Rajasilta, M., Laine, P., Eklund, J., Montesino-Pouzols, F., Corona, F., Junker, K., Meier, H. and Dippner, J. W. (2015). Scenario simulations of future salinity and ecological consequences in the Baltic Sea and adjacent North Sea areas–implications for environmental monitoring. *Ecological Indicators* **50**, 196-205.

Willmer, P., Stone, G. and Johnston, J. (2000). Environmental Physiology of Animals: Blackwell Science.

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology* **208**, 2819-2830.

Zaklan, S. D. and Ydenberg, R. (1997). The body size–burial depth relationship in the infaunal clam *Mya arenaria*. *Journal of Experimental Marine Biology and Ecology* **215**, 1-17.

Zwarts, L. and Wanink, J. (1989). Siphon size and burying depth in deposit- and suspension-feeding benthic bivalves. *Marine Biology* **100**, 227-240.

TABLES

Table 1. Mitochondrial respiratory states and derived indices used to assess the mitochondrial function in *M. arenaria*.

Respiratory state or index	Reflective of:	Determined as:
OXPHOS	OXPHOS capacity	Respiration in the presence of ADP
PL	Proton leak	Respiration in the presence of oligomycin
ETS	ETS capacity	Respiration after the addition of an uncoupler (CCCP)
COX	Capacity of the cytochrome c oxidase	Respiration rates after additions of antimycin A, TMPD and ascorbate
RCR	Respiratory control ratio	=OXPHOS/PL
COX/OXPHOS	Reserve COX capacity	=COX/OXPHOS
ETS/OXPHOS	Reserve ETS capacity	=ETS/OXPHOS

Table 2. ANOVA: Effect of the exercise group, salinity regime and their interaction on burial time and bioenergetics on *Mya arenaria*.

Groups – control, exercised and exhausted. Salinity regimes – normal, low and fluctuating salinity. Size of the clam shell and the number of times the clams were disturbed (ToD) were used as covariates. Proteins, lipids, and carbohydrates content, as well the total energy reserves and ETS capacity were determined in the whole body of the clams after the foot (<1% of the body mass) was removed. F ratios (with degree of freedom and the error shown as a subscript) and P values for the effects are given. Significant effects (P<0.05) are highlighted in bold. ND – not determined.

Parameters	Group	Salinity	Group x Salinity	Shell size	ToD
Burial time	$F_{1,37}$=2.31 P=0.137	**$F_{2,37}$=6.41 P=0.004**	$F_{2,37}$=0.34 P=0.715	**$F_{1,37}$=10.09 P=0.003**	ND
Protein content	**$F_{2,47}$=13.83 P<0.001**	**$F_{2,47}$=4.56 P=0.015**	**$F_{4,47}$=4.54 P=0.003**	$F_{1,47}$=1.27 P=0.265	$F_{1,47}$=2.38 P=0.129
Lipid content	**$F_{2,45}$=11.44 P<0.001**	**$F_{2,45}$=10.40 P<0.001**	$F_{4,45}$=1.10 P=0.369	$F_{1,45}$=3.24 P=0.079	$F_{1,45}$=0.44 P=0.509
Carbohydrate content	$F_{2,50}$=2.39 P=0.102	$F_{2,50}$=1.91 P=0.158	$F_{4,50}$=1.36 P=0.261	$F_{1,50}$=0.53 P=0.470	$F_{1,50}$=1.25 P=0.268
Total body energy reserves	**$F_{2,48}$=17.64 P<0.001**	$F_{2,48}$=2.72 P=0.076	$F_{4,48}$=0.80 P=0.534	$F_{1,48}$=4.04 P=0.050	$F_{1,48}$=3.66 P=0.062
ETS capacity	$F_{2,47}$=2.79 P=0.072	**$F_{2,47}$=12.61 P<0.001**	**$F_{4,47}$=5.06 P=0.002**	$F_{1,47}$=3.50 P=0.068	$F_{1,47}$=2.72 P=0.106
Es/Ec	**$F_{2,46}$=3.51 P=0.038**	**$F_{2,46}$=4.33 P=0.019**	$F_{4,46}$=1.94 P=0.120	$F_{1,46}$=0.57 P=0.454	$F_{1,46}$=1.35 P=0.251

Table 3. ANOVA: Effect of salinity regime, assay osmolarity and their interaction on respiration of the gill mitochondria of *Mya arenaria*.

F ratios (with degree of freedom and the error shown as a subscript) and P values for the effects are given. Significant effects (P<0.05) are highlighted in bold.

Parameters	Salinity	Assay osmolarity	Salinity* Assay osmolarity
RCR	**$F_{2,26}$=4.02 P=0.030**	**$F_{1,26}$=18.82 P<0.001**	$F_{2,26}$=2.92 P=0.072
OXPHOS	$F_{2,26}$=0.68 P=0.517	$F_{1,26}$=1.82 P=0.190	$F_{2,26}$=0.51 P=0.606
PL	$F_{2,26}$=0.06 P=0.940	$F_{1,26}$=0.70 P=0.410	$F_{2,26}$=0.19 P=0.829
COX	$F_{2,26}$=1.47 P=0.248	$F_{1,26}$=2.41 P=0.132	$F_{2,26}$=0.35 P=0.709
ETS	$F_{2,26}$=0.40 P=0.673	$F_{1,26}$=2.66 P=0.115	$F_{2,26}$=0.66 P=0.523
COX/OXPHOS	**$F_{2,26}$=4.16 P=0.027**	$F_{1,26}$=1.40 P=0.247	$F_{2,26}$=1.52 P=0.238
ETS/OXPHOS	$F_{2,30}$=2.86 P=0.073	**$F_{1,30}$=5.18 P=0.030**	$F_{2,30}$=1.07 P=0.355

FIGURES

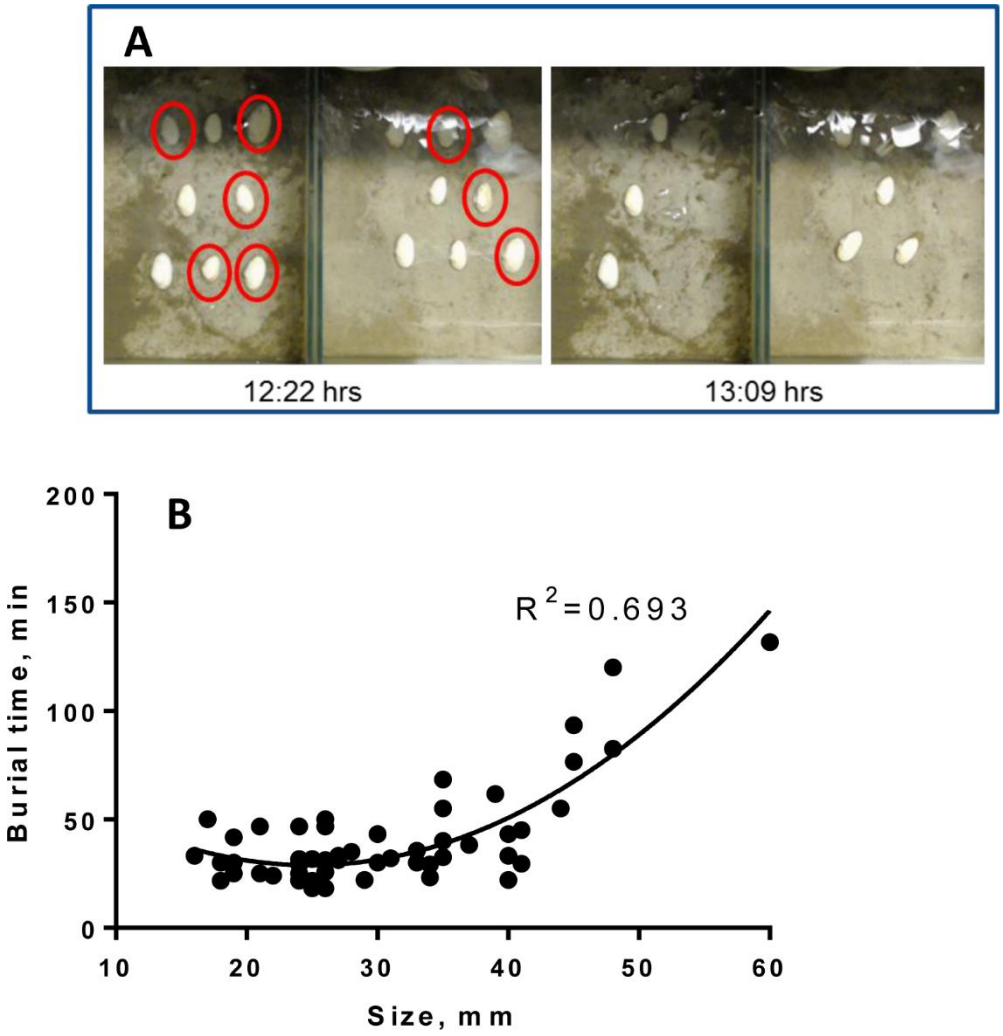

Figure 1. The effects of the clam size on the digging activity of *M. arenaria* measured as the time needed to bury after the disturbance.

A: Representative still frames of the video recording showing clams at the beginning of the burrowing cycle (i.e. immediately after the disturbance) and after 45 min. Clams that completely re-buried after 45 min are marked by the oval shapes in the left still frame. B: Size-dependent activity assessed at the normal salinity (15) in the clams acclimated to the NS regime. The line represents a quadratic regression; the goodness-of-fit is shown in the graph.

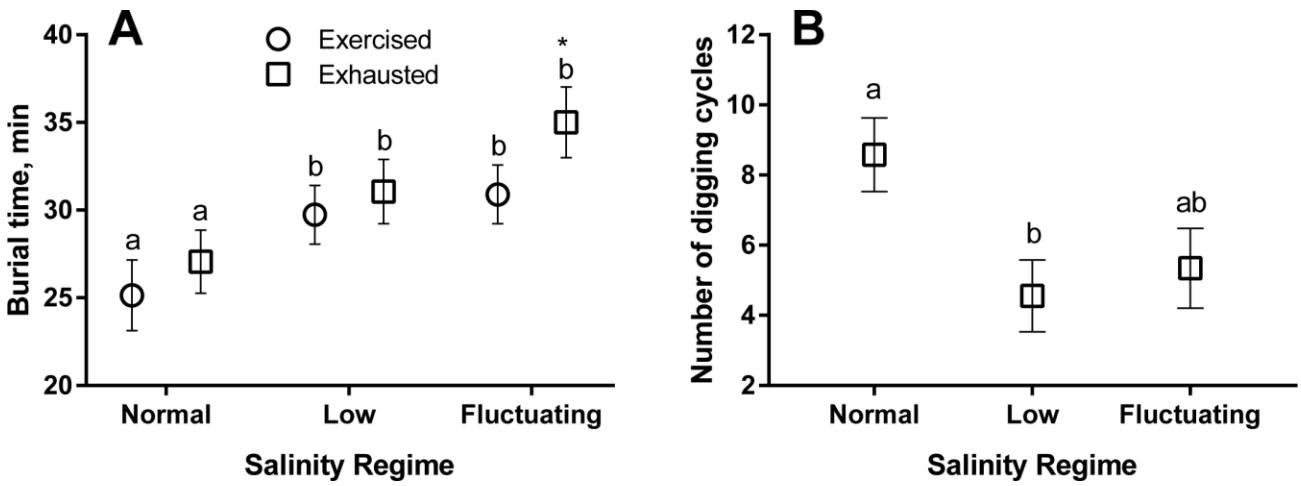

Figure 2. Effect of the acclimation salinity on the digging performance of *M. arenaria*.

A: The mean burial times of clams acclimated at different salinities. B: The average number of times the clams were able to re-bury before exhaustion. The means were corrected to the average clam size across all groups (26 mm and 24 mm in A and B, respectively). Different letters indicate the values that are significantly different among different salinity regimes within the same activity group (exercising or exhausted) ($P<0.05$); if the columns share a letter, the respective values are not significantly different ($P>0.05$). Asterisks indicate the values that are significantly different between exercising and exhausted group at the same salinity regime ($P<0.05$). N= 6-8.

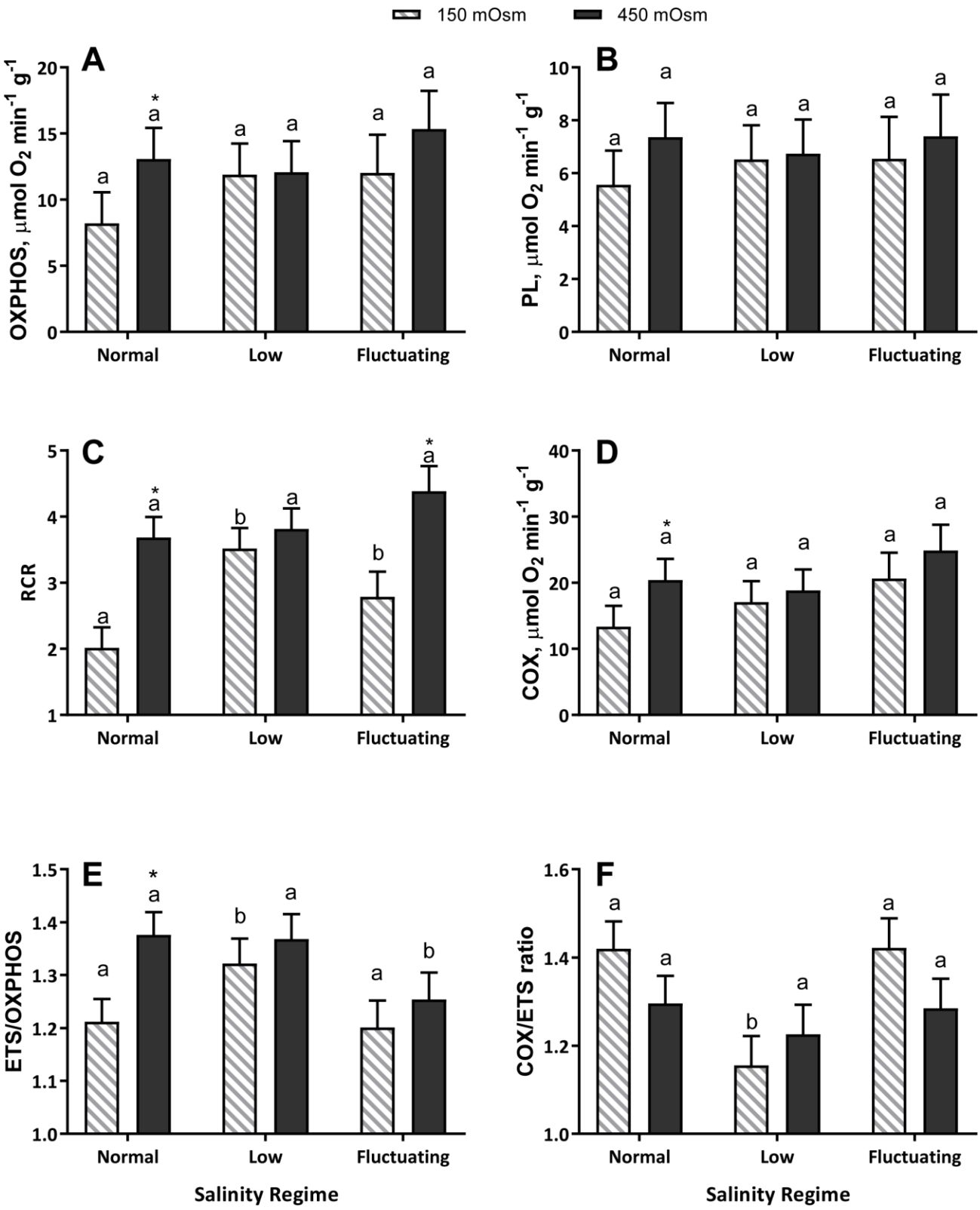

Figure 3. Effect of high (450 mOsm) and low (150 mOsm) osmolarity on the mitochondrial functions in the gills of clams acclimated at the different salinities.

A – OXPHOS capacity, B – proton leak rate, C – respiratory control ratio (RCR), D – cytochrome C oxidase activity, E –apparent reserve ETS capacity, and F – apparent reserve COX capacity. Different letters indicate the values that are significantly different among clams acclimated at the different salinity regimes ($P<0.05$). Asterisks indicate the values that are significantly different between mitochondria measured al high and low osmolarity within the same salinity acclimation group ($P<0.05$). N=4-7.

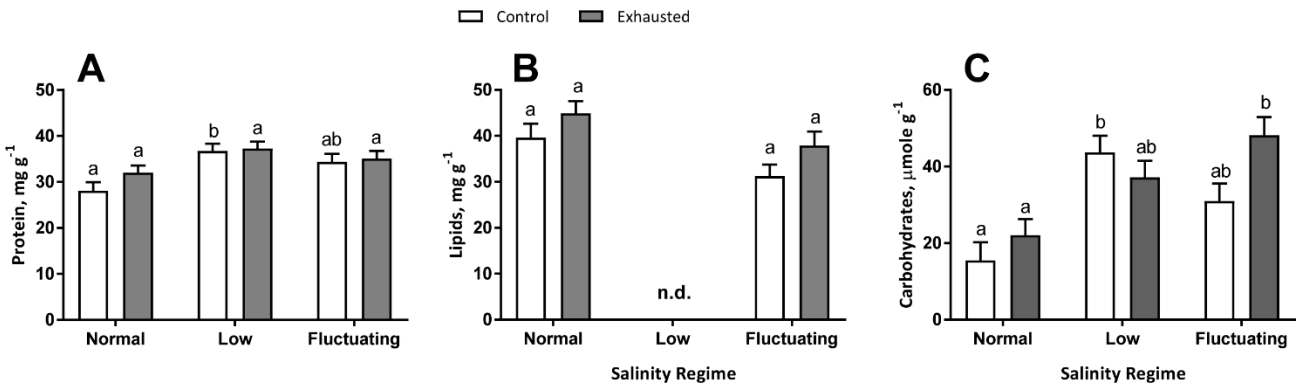

Figure 4. Effects of the salinity regime and digging on energy reserves in the foot of *M. arenaria*.

A – proteins, B – lipids, and C – carbohydrates in the foot of *M. arenaria* calculated per g wet mass. The means were corrected for the average clam size (26-27 mm) for all traits. Different letters indicate the values that are significantly different among the salinity regimes within the same activity group (control or exhausted); if the columns share a letter, the respective values are not significantly different ($P>0.05$). Asterisks indicate the values that are significantly different from the control group at the same salinity regime ($P<0.05$). Data for the lipid content in the clams acclimated to the low salinity are missing due to the sample loss (n.d.). N= 6-8.

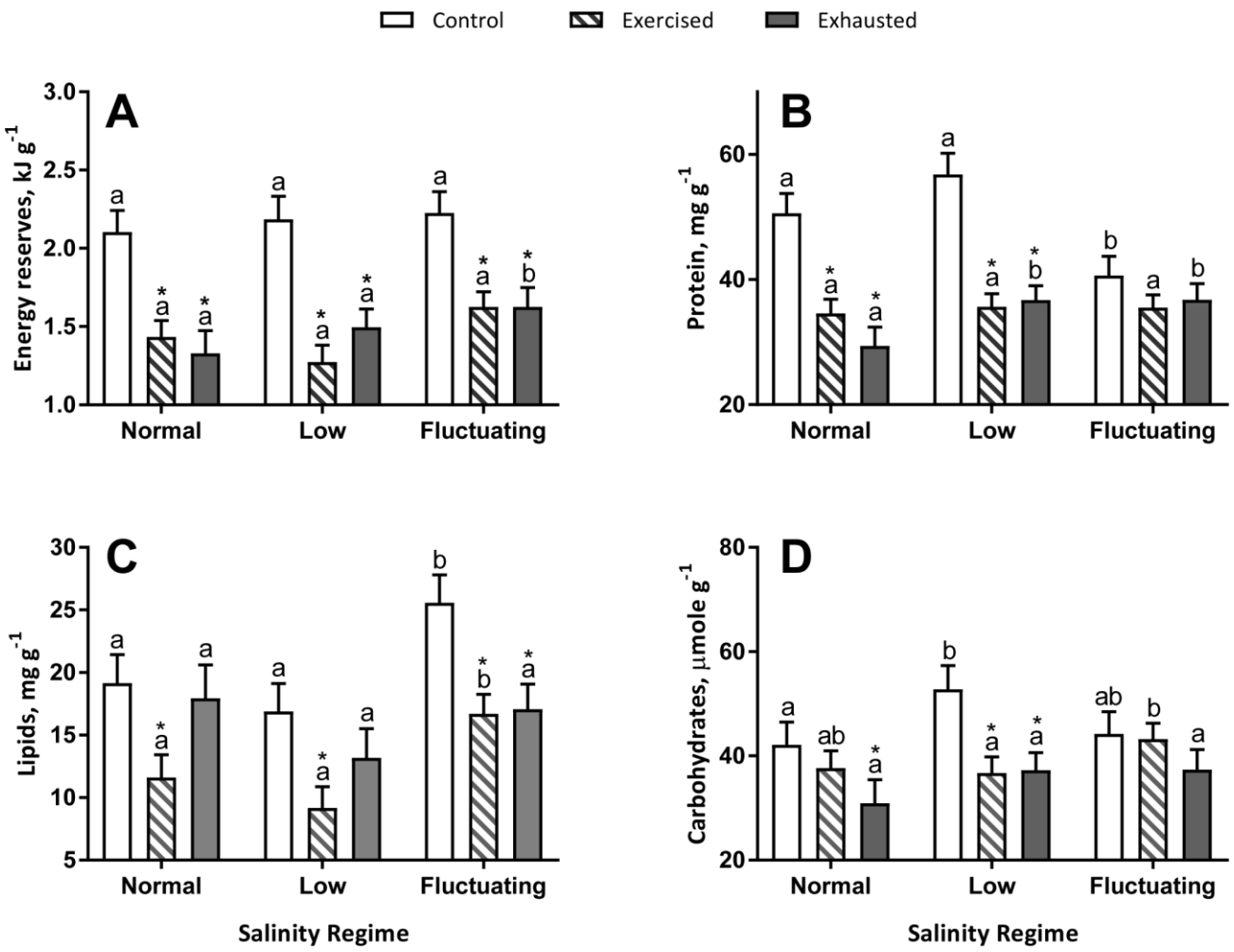

Figure 5. Effects of the salinity regime and repeated digging on the total body energy reserves of *M. arenaria*.

A – total energy reserves, B – proteins, C – lipids, and D – carbohydrates of *M. arenaria* calculated per g wet body mass. The means were corrected for the average clam size (27 mm) and the average times of disturbance (4) for all traits. Different letters indicate the values that are significantly different among salinity regimes within the same activity group (control, exercising or exhausted); if the columns share a letter, the respective values are not significantly different ($P>0.05$). Asterisks indicate the values that are significantly different from the control group at the same salinity regime ($P<0.05$). N= 4-8.

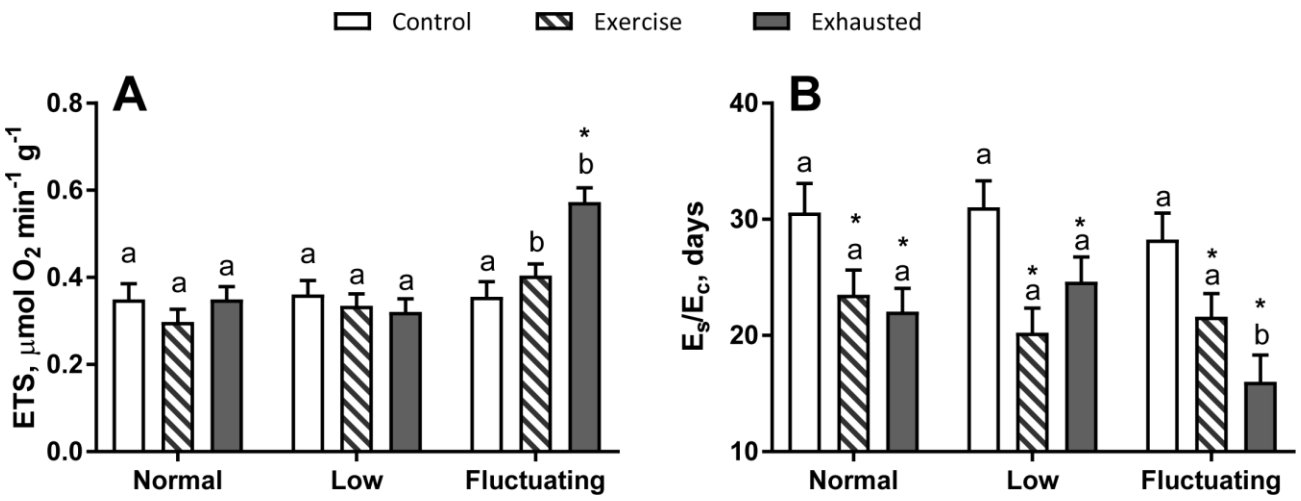

Figure 6. Effects of the salinity regime and repeated digging on the whole-body aerobic capacity of *M. arenaria*.

A – The whole-body aerobic capacity was assessed as the ETS activity, and B – the ratio of available energy (energy stored, E_s) to consumed energy (ETS capacity, E_c) corrected to a common clam size (27 mm). Different letters indicate the values that are significantly different among salinity regimes within the same activity group (control, exercising or exhausted); if the columns share a letter, the respective values are not significantly different ($P>0.05$). Asterisks indicate the values that are significantly different from the control group at the same salinity regime ($P<0.05$). N=5-8.

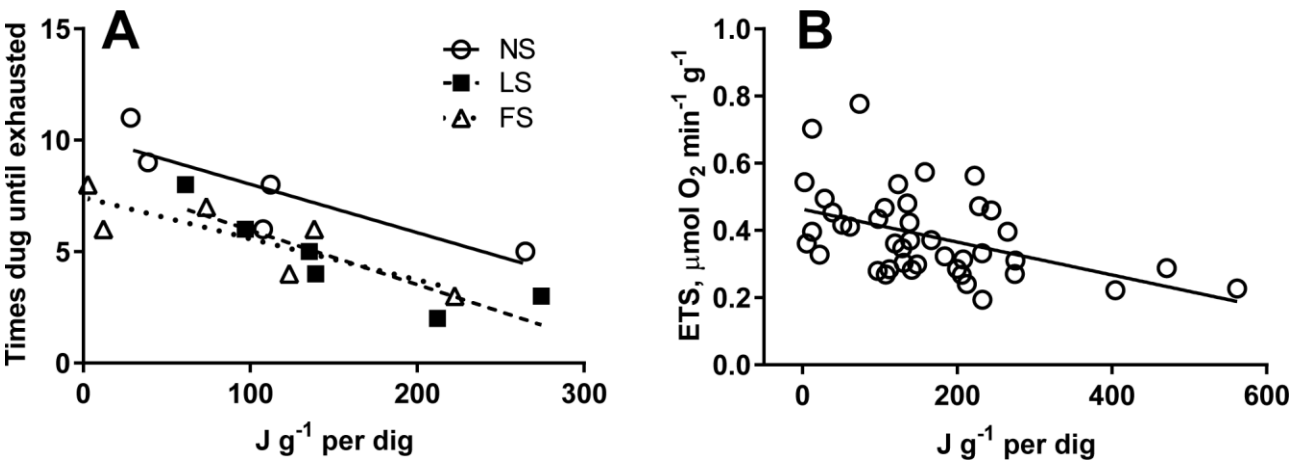

Figure 7. The relationship between the ETS capacity, burying performance and the energy cost of burying in *M. arenaria*.

A –Relationship between the amount of times a clam was able to re-bury prior to exhaustion and the amount of energy spent for one dig. NS – normal salinity, LS – low salinity, FS – fluctuating salinity. The slopes of the lines from clams acclimated at the different salinities are not significantly different ($P>0.05$). The intercepts of the regression line for the NS group is significantly different from those for the LS and FS groups ($P<0.05$). N=5-6.

B – Relationship between the whole-organism ETS capacity and energy expenditure per dig for exercising and exhausted clams. The slopes and intercepts of the regression lines for the clams acclimated at different salinities were not significantly different ($P>0.05$), and therefore, a single regression was constructed for all data. N=42.