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Size- and age-dependent changes in adductor muscle swimming physiology of the scallop *Aequipecten opercularis*

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SUMMARY

The decline of cellular and especially mitochondrial functions with age is, among other causes, held responsible for a decrease in physiological fitness and exercise capacity during lifetime. We investigated size- and age-related changes in the physiology of exercising specimens of the short lived swimming scallop *Aequipecten opercularis* (maximum life span 8 to 10 years) from the Isle of Man, UK. *A. opercularis* swim mainly to avoid predators, and a decrease in swimming abilities would increase the risk of capture and lower the rates of survival. Bigger (older) individuals were found to have lower mitochondrial volume density and aerobic capacities (citrate synthase activity and adenylates) as well as less anaerobic capacity deduced from the amount of glycogen stored in muscle tissue. Changes in redox potential, tissue pH and the loss of glutathione in the swimming muscle during the exercise were more pronounced in young compared to older individuals. This indicates that older individuals can more effectively stabilize cellular homeostasis during repeated exercise than younger animals but with a possible fitness cost as the change in physiology with age and size might result in a changed escape response behaviour towards predators.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/15/2492/DC1

Key words: ageing, bivalves, exercise, ROS.

INTRODUCTION

Ageing is a multifactorial process which involves progressive deterioration of cells, tissues and organs that altogether causes a decline in physiological functioning in the whole organism over their life time. A major paradigm of cellular aging holds the life long production of reactive oxygen species (ROS) in aerobic metabolism responsible for damaging cell constituents such as lipids, proteins and DNA and altering the function of these biomolecules. We have been investigating ROS production and turnover in bivalve molluscs for several years now and can track the progressive accumulation of ROS-mediated damage and decline of cellular function with age in different bivalve species (Philipp et al., 2005a; Philipp et al., 2006; Philipp et al., 2005b; Strahl et al., 2007). This led us to conjecture that the constraints in cellular and metabolic functioning posed by the aging process may also impair the physiological response capacity towards environmental stress in aged individuals at the whole organism level. Bivalves prove to be attractive models to study ageing for several reasons. They are ectotherms, they thrive in considerably diverse habitats and have thus evolved different life styles, aside from their shells providing a readable record of their chronological age. Thus, in contrast to other classical ageing models such as rats, mice, *D. melanogaster* and *C. elegans*, it is possible to compare bivalves from various environments and determine their individual chronological age and natural maximum life span in each local population. This information can be further used to establish and combine the influence of intrinsic (genetic) and extrinsic/environmental (e.g. predation pressure) factors on the ageing process.

Mitochondria are centrally involved in the process of cellular aging and extremely vulnerable to ROS damage. Their membranes contain a high degree of unsaturated fatty acids and the mitochondrial DNA lies, unprotected by histones, in close vicinity to the ROS generating respiratory chain systems of the inner mitochondrial membrane.

Metabolic rate can influence ROS generation rate (for reviews, see Jackson, 2005; Sen and Packer, 2000), thus, an exercise-induced increase of metabolic oxygen turnover may possibly be connected to increased ROS generation, leading to mitochondrial damage. However, during moderate exercise, ROS generation rates may even become reduced as mitochondria enter state three (substrate and ADP available), where the lowest possible membrane potential and the lowest ROS generation rate have been found to occur (Philipp et al., 2005b). Accordingly, the changes in ROS formation obtained by using exercise studies are diverse (Jackson, 2005). Studies on mammals for example, using different levels of treadmill running (exhaustive, moderate, low) resulted in increased, no, or even decreased lipid peroxidation (malondialdehyde accumulation) (Lovlin et al., 1987).

In the present study we investigated the effects of exercise physiology on physiological ageing in the scallop *Aequipecten opercularis*. The queen scallop *Aequipecten opercularis* from the Isle of Man, UK is short lived [8 to 10 years (Ansell et al., 1991; Philipp et al., 2006)] and belongs to an active swimming ecomorph within the scallop group (Minchin, 2003). These animals actively swim and change their location to avoid unfavourable environmental conditions including the escape from predators (Paul, 1980; Wong and Barbeau, 2003). A decline in physiological fitness, e.g. exercise capacity with age, has been reported for humans, rat and mice and has been related to a decline in

mitochondrial function and mitochondrial volume density with age, as there is a positive correlation between mitochondrial function and volume density to fatigue resistance and exercise capacity (Creed et al., 2004). Decreasing swimming capacity with age could lead to a higher risk of capture and lower survival rate of aged individuals.

In a previous study investigating clap rates and swimming, as well as valve closure behaviour, we examined the swimming capacity in smaller and bigger *A. opercularis* individuals. We observed no difference in clap rate, but found that nearly 50% of young scallops closed their shells completely and for as long as 30 min following the predator attack. By contrast, just 3% of the bigger individuals behaved in this way and the majority remained permanently open or opened the shells again within the 30 min experimental observation (Schmidt et al., 2008).

Based on these behaviour experiments, we assumed a change of cellular physiology to occur in *A. opercularis* with size and age, which might involve a decrease in anaerobic and aerobic energy generating capacities, caused by a decline of mitochondrial function and/or volume density with size and age. Therefore, in the present study we investigated components of the aerobic and anaerobic energy generating systems, as well as redox potential, antioxidant defence systems and markers of oxidative damage during exercise in the phasic (striated) adductor muscle of smaller (younger) and bigger (older) individuals, to see whether the observed change in swimming behaviour with size is at least partly due to physiological changes.

MATERIALS AND METHODS

Sampling and maintenance

Irish Sea queen scallops, *Aequipecten opercularis* Linnaeus 1758, were dredged 12 miles south of Port St Mary, Isle of Man in July 2005 at about 51 m water depth. Animals were transported to the Port Erin Marine Laboratory and kept in natural sea water flow-through aquaria at ~14°C and 34 PSU for several days. Animals were transported in thermoboxes with wet cotton wool and supplemented with oxygen and cold packs to the Alfred-Wegener-Institute of Polar and Marine Research, Germany. *A. opercularis* individuals were kept in flow-through aquaria at ~10°C (mean *in situ* temperature) and 34 PSU for 2 weeks prior to experimentation. Animals were fed twice a week with life plankton (DT's Live Marine Phytoplankton®, Sycamore, IL, USA).

Individuals were be grouped in two classes: small animals from 40–55 mm (below marketable size) (Jenkins et al., 2003) and bigger animals from 65–75 mm shell height. Age was determined with the von Bertalanffy growth function (VBGF) of Philipp et al. (Philipp et al., 2006) obtained for *A. opercularis* from the same sampling station. The mean age of the small animals was 2±0.5 years (mean ± s.e.m.) and for the big animals it was 4±0.5 years.

Swimming experiments

Experimental design

Scallops were fixed with a Teflon screw within the experimental setup and the whole system was video recorded as described by Schmidt et al. (Schmidt et al., 2008) (see Supplementary material Movie 1).

Swimming was triggered by the sea star *Asterias rubens*, a natural predator in the environment of *A. opercularis*. The swimming experiment (Table 1) consisted of a 30 min acclimation phase to experimental conditions (unstressed animals, group 0), run 1: 1 min predator stress (group 1) and 15 min recovery (group 2), run 2: again 1 min predator stress (group 3) and 15 min recovery (group 4). During every 1 min swimming period *A. opercularis* individuals were touched up to ten times by the sea star even if the shell was closed. Directly after the respective event, individuals of each group were removed from the experimental aquaria quickly dissected and the adductor muscle snap frozen in liquid nitrogen for biochemical analysis.

Isolation of adductor muscle mitochondria

For identification of general function of adductor muscle mitochondria of unstressed *A. opercularis*, state 2 and 3 respiration with succinate or glutamate as energetic substrates, and H_2O_2 generation in state 2 were investigated in 16 mitochondrial isolates. Mitochondria were isolated from the muscle tissue of freshly sacrificed bivalves, ranging in size from 51.5–70 mm shell height, using the method of Guderley et al. (Guderley et al., 1995).

Tissues of one or two *A. opercularis* specimens were pooled for one experiment. About 3 g of muscle tissue were finely chopped in four to five times the volume of homogenisation buffer (480 mmol l^{-1} sucrose, 30 mmol l^{-1} Hepes, 230 mmol l^{-1} KCl, 3 mmol l^{-1} Na_2-EDTA, 6 mmol l^{-1} EGTA, 5 mmol l^{-1} $MgCl_2$, 1% bovine serum albumin, 1 µl ml^{-1} aprotinine, pH 7.0). Briefly, the tissue was homogenised in a pre-cooled glass and Teflon homogeniser, centrifuged at 900 ***g*** for 10 min at 2°C and the supernatant collected. The supernatant was centrifuged at 10 000 ***g*** for 10 min to sediment the mitochondria. The resulting pellet was resuspended in 1 volume of isolation medium without $MgCl_2$ and centrifuged at 10 000 ***g*** for 10 min. The final pellet was resuspended in magnesium-free isolation medium containing 1% BSA (1 g 100 ml^{-1}) and 1 µg ml^{-1} aprotinine.

Respiration of isolated mitochondria

Mitochondrial respiration was measured at 10°C in respiration buffer (480 mmol l^{-1} sucrose, 70 mmol l^{-1} Hepes, 158 mmol l^{-1} KCl, 10 mmol l^{-1} KH_2PO_4, 50 mmol l^{-1} taurine, 50 mmol l^{-1} β-alanine, pH 7.4). The substrate used was 24 mmol l^{-1} succinate, with 5 µmol l^{-1} rotenone to prevent respiration of endogenous NAD-linked substrates (Brand, 1995), or 29 mmol l^{-1} glutamate, and state 3 respiration was induced by addition of 0.6 mmol l^{-1} ADP. Respiration rate was recorded with oxygen microoptodes® (PreSens GmbH, Neuweiler, Germany). Oxygen concentrations were calculated using the oxygen solubility (βO_2) according to Johnston et al. (Johnston et al., 1994) and the atmospheric pressure of the day.

Production of hydrogen peroxide (H_2O_2) by isolated mitochondria

Mitochondrial hydrogen peroxide production was measured fluorimetrically by recording the reaction of H_2O_2 with homovanilic

Table 1. Group allocation in the swimming experiment

	Run 1		Run 2	
30 min acclimation	Sea star attacks 1 min	15 min recovery	Sea star attacks 1 min	15 min recovery
= group 0	= group 1	= group 2	= group 3	= group 4

acid (HVA; $\lambda_{exitation}$=312 nm and $\lambda_{emission}$=420 nm) in the presence of horseradish peroxidase (HRP), using a procedure modified after Miwa et al. (Miwa et al., 2003). The H_2O_2 generation rate of *A. opercularis* mitochondria was measured with a LS 50B Perkin Elmer fluorometer with a cooled sample compartment and magnetic stirring. The H_2O_2 generation in state 2 was recorded for each mitochondrial aliquot directly in the fluorometer. A subsample of 150 µl *A. opercularis* mitochondrial solution was incubated with 850 µl assay medium to which 0.1 mmol l^{-1} HVA and 2.5 i.u. ml^{-1} HRP at 10°C were added. As soon as the fluorescence signal steadied, the following chemicals were added in the order: (1) 24 mmol l^{-1} succinate, 5 µmol l^{-1} rotenone, 100 i.u. superoxide dismutase (SOD), 20 µmol l^{-1} antimycin; (2) 29 mmol l^{-1} glutamate, 100 i.u SOD, 20 µmol l^{-1} antimycin. Alternatively, in the order glutamate, antimycin, SOD, where rotenone is an inhibitor of respiratory complex I and antimycin an inhibitor of respiratory complex III.

In each experiment, fluorescence was calibrated with an H_2O_2 standard (0.2 nmol l^{-1}; Merck, Darmstadt, Germany). Both, H_2O_2 generation rates and oxygen consumption rates were measured in parallel and related to mitochondrial protein content (see Keller et al., 2004).

Mitochondrial density

For assessment of mitochondrial density a total of 14 (seven young and seven old) samples of the striated adductor muscle were dissected, immersion-fixed in Karnovsky's (Karnovsky, 1965) paraform–aldehyde–glutaraldehyde fixative, post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanols and embedded in resin.

Ultrathin sections were cut on a Reichert-Jung (Vienna, Austria) Ultracut microtome and mounted on Formvar-coated 75- and 100-mesh copper grids. The sections were then contrasted with aqueous solutions of uranyl acetate and lead citrate and viewed in a Zeiss EM 910 transmission electron microscope (see Fig. 1, TEM). Based on randomised photosampling, 40 micrographs per muscle tissue sample were taken and the volume densities of mitochondria per muscle fibre V_V(Mito/Mf) were determined using stereological methods (Weibel, 1979).

After testing, normality and equal variances differences between the two age groups were assessed with a two-sided unpaired *t*-test (Sigma Stat 3.1).

Biochemical measurements

Citrate synthase (EC 4.1.3.7)

Frozen muscle tissue of control animals was ground in liquid nitrogen and homogenised with a glass homogeniser (Nalgene, Rochester, NY, USA) in Tris–HCl buffer [20 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, 0.1% (v/v) Tween 20, pH 7.4] 1:10 (w/v). Homogenates for citrate synthase (CS) activity were sonicated for 15 min in a Branson Sonifier 450 (output control 8, duty cycle 50%) cooled to 0°C and centrifuged at 7400 ***g*** for 5 min at 2°C. CS activity was measured after Sidell et al. (Sidell et al., 1987) recording the absorbance increase of 0.25 mmol l^{-1} DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] in 75 mmol l^{-1} Tris–HCl (pH 8.0), 0.4 mmol l^{-1} acetyl-CoA and 0.4 mmol l^{-1} oxaloacetate at 412 nm. Activity was calculated using the millimolar extinction coefficient ε_{412} mmol l^{-1} of 13.61 mmol l^{-1} cm^{-1}.

ATP, ADP and AMP

Adenylate concentrations were measured after Lazzarino et al. (Lazzarino et al., 2003) using high performance liquid chromatography (HPLC).

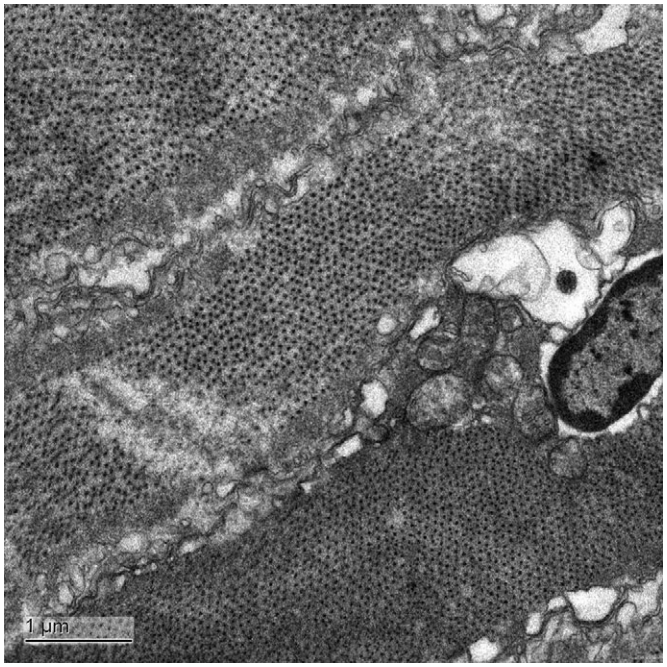

Fig. 1. Transmission electron microscope image of an *A. opercularis* adductor muscle with visible mitochondria in the middle-right part of the picture.

Frozen muscle tissue was ground in liquid nitrogen and homogenised with a micropistill in a 1.5 ml reaction vial with ice-cold, nitrogen saturated precipitation solution [CH_3CN (acetonitrile) + 10 mmol l^{-1} KH_2PO_4, at a ratio of 3:1, pH 7.4] at a 1:10 (w:v) tissue to medium ratio. Precipitation solution was prepared weekly and the pH checked immediately before use. The homogenate was centrifuged at 20 690 ***g*** for 10 min at 4°C and the clear supernatants were stored on ice. Pellets were supplemented with 1 ml of the precipitation solution and resuspended for several seconds using an ultraturrax, centrifuged again as above, and the supernatants combined. This extract was washed with the double volume of chloroform (10 sec vortexing with HPLC grade CH_3Cl) and centrifuged as above. The upper aqueous phase, containing the water-soluble low molecular mass compounds, was collected and washed again twice with chloroform. Supernatants were then stored at –80°C until measurement.

Samples were separated by HPLC using a Kromasil 250 mm×4.6 mm, 5 µm particle size column (Eka Chemicals, AB, Bohus, Sweden) and its own guard column. Injection volume was 50 µl of undiluted extract. HPLC conditions (solvents, gradient, flow rate, detection) were applied as described in Lazzarino et al. (Lazzarino et al., 2003). ATP, ADP and ATP standards were purchased from Sigma. Calibration and calculation of adenylate concentration in the samples were done using Karat Software 7.0 (Beckman Coulter GmbH, Krefeld, Germany). Energy charge (EC) was calculated after Atkinson (cf. Ataullakhanov and Vitvitsky, 2002):

$$EC = (ATP + ADP/2) / (ATP + ADP + AMP) .$$

The adenylate pool was calculated after Ataullakhanov and Vitvitsky (Ataullakhanov and Vitvitsky, 2002):

$$\text{Adenylate pool} = ATP + ADP + AMP .$$

Glycogen

Glycogen concentration was determined after Kunst et al. (Kunst et al., 1984) and Keppler and Decker (Keppler and Decker, 1974). Muscle tissue (20–50 mg) was ground in liquid nitrogen, 1 ml ice-cold Milli-Q water added and the sample sonified on ice at 30% output control (Branson Sonifier Cell disruptor B15; Danbury, CT, USA). The homogenate was incubated for 10 min at 95°C for protein denaturation. To hydrolyse glycogen to glucose, 250 µl of the homogenate was mixed with 500 µl acetate buffer (0.1 mol l^{-1}, pH 4.8) and 20 µl amyloglucosidase (Roche, Mannheim, Germany) and incubated for 2 h at 40°C. The rest of the homogenate was kept on ice for later determination of the free glucose concentration.

After incubation, both samples were centrifuged at 15 000 ***g*** for 10 min at 4°C. The supernatant was saved for glucose determination and measured, using the glucose determination kit (D-glucose UV test, r-biopharm, Darmstadt, Germany) at 340 nm in the photometer. A standard curve was prepared using the standard solution of the kit.

Glutathione content, intracellular pH, redox potential

The concentrations of glutathione in the oxidised (GSSG) and reduced (GSH) forms were measured after Fariss and Reed (Fariss and Reed, 1987), using high performance liquid chromatography (HPLC). The principle of the measurement is the derivatisation of the thiols with dinitrofluobenzene (DNFB). GSH oxidation during extraction is prevented by iodoacetic acid (IAA) binding of GSH. Tissues were ground in liquid nitrogen and homogenised with ice-cold perchloric acid (PCA; 10% with 2 mmol l^{-1} bathophenanthrolinedisulfonic acid; bpDS) at 1/10 (w/v) bubbled with nitrogen prior use. Following centrifugation at 15 000 ***g*** and 4°C for 5 min, 500 µl of the supernatant was transferred to a fresh reaction vial and 10 µl of the pH indicator (1 mmol l^{-1} m-Cresol Purple in H_2O with 0.5 mol l^{-1} iodoacetic acid) and 50 µl internal standard (1 mmol l^{-1} gamma-glutamyl-glutamate in 0.3% PCA) added. Samples were titrated to pH 8.5 with 4 mol l^{-1} KOH with 0.3 mol l^{-1} n-morpholinopropanesulfonic acid and incubated for 45 min at room temperature to allow iodoacetic acid to bind the GSH. After 5 min centrifugation at 15 000 ***g*** and 4°C, 1% DNFB (1% 1-fluoro-2.4-dinitrobenzene) in ethanol was added to the supernatant at a 1:3 ratio and incubated in a dark vial for 24 h at room temperature without shaking. Standards of GSH and GSSG were prepared in 10% PCA with bpDS and treated as samples.

Prior to injection into the HPLC, thawed samples were again centrifuged for 1 min at 7500 ***g*** and 4°C, to remove the remaining PCA and the supernatant filtered through a 0.2 µm nylon membrane filter. Samples were transferred to dark autosampler vials and injected using an autosampler that was thermostatted to 4°C. Separation was achieved on a NH_2-spherisorb column (240×4 mm, 5 µm particles) and its own guard column at 39°C using a binary solvent system of A: 80% methanol–water, and B: 80% solvent A and 20% acetate stock (272 g sodium acetate trihydrate diluted in 122 ml water plus 378 ml glacial acetic acid). Both solvents were degassed and filtered (0.45 µm pore size) prior to use. Flow rate was 1.2 ml min^{-1} at a maximal backpressure of 2500 psi. The gradient program was: 90% A, 10% B for 12 min followed by 30 min of linear gradient elution to 45% A, 55% B and a subsequent 8 min hold. Thereafter the system was returned to the initial conditions within 5 min and re-equilibrated for 15 min.

Tissue pH

Tissue pH (pH_i) was determined using the homogenate technique (Pörtner et al., 1990) in a system thermostated at 10°C, the maintenance temperature of the scallops.

Prior to measurements, the pH electrode (SenTix Mic, WTW, Weilheim, Germany) was calibrated at the *A. opercularis in situ* temperature of 10°C with precise calibration solutions (AppliChem, Darmstadt, Germany; pH 6.865 – A1259; pH 7.413 – A1260). The pH were recorded on a Kipp and Zonen chart recorder.

For tissue measurements, muscle tissue (100–150 mg) was ground in liquid nitrogen and the powder added to an Eppendorf tube containing 0.3 ml of medium composed of 160 mmol l^{-1} potassium fluoride, 2 mmol l^{-1} nitrilotriacetic acid. The tube was closed after layering with air-bubble-free medium, and the tissue homogenised by ultrasound (Branson, sonifier 450, duty cycle 40%, output control 8) at 0°C and centrifuged at 20 000 ***g*** at 10°C for 30 s. The pH in the supernatant was determined in the system thermostatted at 10°C.

Glutathione concentrations and corresponding pH_i values of each sample were used to calculate the tissue redox potential after Schafer and Büttner (Schafer and Büttner, 2001).

Catalase activity

Catalase activity was determined after Aebi (Aebi, 1984). Frozen tissue (100–150 mg) was ground in liquid nitrogen and homogenised with a micropistill in 50 mmol l^{-1} phosphate buffer (50 mmol l^{-1} KH_2PO_4, 50 mmol l^{-1} Na_2HPO_4, pH 7.0) with 0.1% Triton X-100 at 1:5 (w/v). Samples were centrifuged at 13 000 ***g*** for 15 min at 4°C. The activity was determined by recording the time of H_2O_2 decomposition, resulting in a decrease of absorption from 0.45 to 0.4 at 240 nm (1 unit).

Lipidperoxidation

Malondialdehyde

Malondialdehyde (MDA) concentrations were measured after Uchiyama and Mihara (Uchiyama and Mihara, 1978). Muscle tissue (200 mg) was ground in liquid nitrogen and homogenised in a glass potter with 0.2% phosphoric acid (bubbled with nitrogen) 1:6 (w/v). The same amount of 2% phosphoric acid was added to achieve a final concentration of 1.1% phosphoric acid and homogenised again. 0.3 ml of the homogenate was transferred to a glass vial and 0.3 ml 1% thiobarbituric acid (TBA) solution added (0.5 g TBA in 50 mmol l^{-1} NaOH + 0.5 ml 10 mmol l^{-1} BHT + 0.2 ml 7% PCA). As a blank, 0.3 ml 3 mmol l^{-1} HCl was added to 0.3 ml sample. The pH of samples and blanks was adjusted to 1.6. Samples were incubated at 100°C for 60 min. After cooling, 1.5 ml butanol was added to each sample and blank and the mixtures vortexed for 40 s. Samples were centrifuged for 5 min at 1000 ***g*** at room temperature. MDA concentrations were measured in the TBA-containing butanol phase at 532 and 600 nm.

Lipidhydroperoxides

The concentrations of lipidhydroperoxides [LOOH; cumene hydroperoxides (CHP) equivalents] were measured after Hermes-Lima et al. (Hermes-Lima et al., 1995), modified for microwell plates. Muscle tissue (100–200 mg) was ground in liquid nitrogen and homogenised in a glass potter with 100% nitrogen-bubbled methanol (1:5–1:10 w/v) and centrifuged for 5 min at 1000 ***g*** and 4°C. Each microwell contained 75 µl $FeSO_4$ (1 mmol l^{-1}), 30 µl H_2SO_4 (0.25 mmol l^{-1}), 30 µl xylenol orange (1 mmol l^{-1}) and 160.5 µl distilled water. 4.5 µl sample was added and incubated for 3 h at room temperature and absorbance measured at 580 nm (E1). For blank subtraction, the sample amount was substituted with distilled water. After determination of E1, 1.5 nmol CHP was added to each sample as an internal standard, and extinction recorded again after 40 min (E2). LOOH concentrations were then calculated as

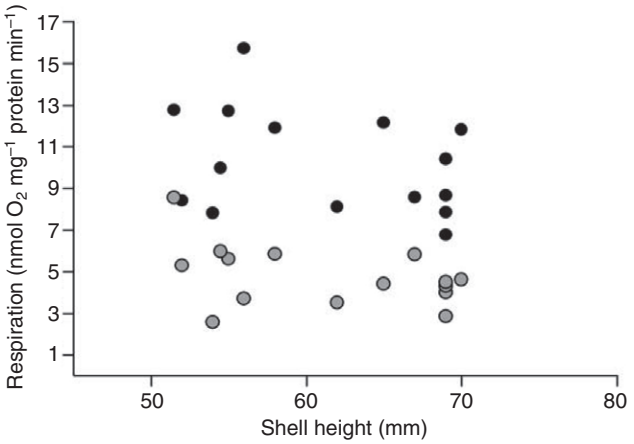

Fig. 2. Respiration of isolated mitochondria of phasic adductor muscle of *A. opercularis* in state 3 (black circles) and state 4 (grey circles). Each dot represents the mean of 1–3 replicate measurements of a single mitochondrial isolation.

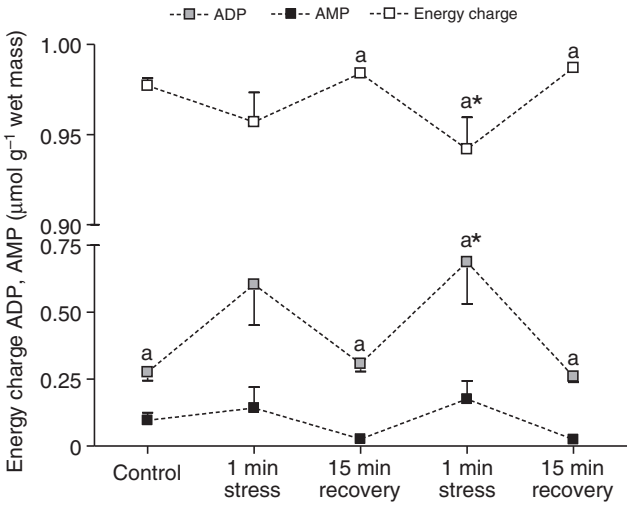

Fig. 3. Data of energy charge (white squares), ADP (grey squares) and AMP (black squares) measured in muscle tissue of both the smaller and bigger individuals, in the different experimental groups (N=12–22). Groups with the same letter are significantly different from each other (P<0.05). If more than two groups have the same letter, the group marked with an asterisk is significantly different to all other groups.

CHP equivalents and expressed as mg^{-1} tissue wet mass, according to Hermes-Lima et al. (Hermes-Lima et al., 1995).

Statistical analysis

To identify changes of respiration and ROS generation of isolated mitochondria with size, a linear regression analysis was used (GraphPad Software). To evaluate the effect of age and groups for glutathione, adenylates, LOOH and MDA, a full factorial covariance model (ANCOVA) was used: dependent parameter *versus* age and group and age × group. ANOVA and *post-hoc* tests were applied to determine significant differences between groups at a significance level of at least P<0.05 (SAS software JMP 5.0.1a).

For CS and glycogen analysis, a two-tailed unpaired *t*-test was used to investigate differences between groups using GraphPad Software 4.0. If data did not show Gaussian distribution, differences were tested using the non parametric Mann–Whitney test. Data that showed different variances were investigated for differences between groups using the unpaired *t*-test with Welch's correction. Outliers were identified using the ESD method (GraphPad Software). All data are presented as mean values ± standard errors unless specified otherwise.

RESULTS

Mitochondrial state 3 and 4 respiration using glutamate as substrate did not change significantly with size and age in the chosen size range (51–70 mm). A slight decrease could be observed but was not significant (Fig. 2). Mean respiration rates (±s.e.m.) were 10.3 (0.65) nmol O_2 mg^{-1} protein min^{-1} in state 3 and 4.7 (0.39) nmol O_2 mg^{-1} protein min^{-1} in state 4.

Table 2. Mitochondrial volume densities and CS activities of the striated adductor muscles of the two sizes of scallops

	Small/young individuals (N)	Big/old individuals (N)	P-value*
V_V(Mito/Mf)	1.270±0.117 (7)	0.922±0.033 (7)	≤0.01
CS (i.u. g^{-1} wet mass)	2.271±0.083 (10)	1.581±0.1082 (10)	≤0.01

V_V(Mito/Mf), mitochondrial volume densities; CS (i.u. g^{-1} wet mass), citrate synthase activity.
Mean size of scallops: smaller, 49.29±6.02 mm for V_V(Mito/Mf) and 51.11±2.2 mm for CS; bigger, 68.0±1.91 mm for V_V(Mito/Mf) and 68.0±1.91 mm for CS.
*Two-sided unpaired *t*-test.

H_2O_2 generation rates in state 2 with either succinate (N=5) or glutamate (N=16) was close to the detection limit with 0.0019 (0.0010) nmol H_2O_2 mg^{-1} protein min^{-1} for succinate and 0.0013 (0.0007) nmol H_2O_2 mg^{-1} protein min^{-1} for glutamate. After addition of antimycin and SOD, H_2O_2 generation increased to values of 0.028 (0.0036) nmol H_2O_2 mg^{-1} protein min^{-1} for glutamate respiration and 0.013 (0.003) nmol H_2O_2 mg^{-1} protein min^{-1} for succinate – rotenone respiration.

Mitochondrial volume density was significantly lower in phasic adductor muscle of bigger compared with smaller individuals (Table 2), which corresponds perfectly with the lower citrate synthase activities found in bigger compared with smaller specimens (Table 2).

ADP concentrations and energy charge (EC) were not significantly different between smaller and bigger individuals but showed significant differences between the experimental groups (two-way ANOVA). For further analysis, data of smaller and bigger individuals were combined for each experimental group (Fig. 3). An increase in ADP and AMP and a decrease in EC were found in both stress groups (groups 1 and 3) compared with the control (group 0) and recovery groups (group 3 and 4; Fig. 3). All parameters showed either a decrease or an increased during exercise and returned to control levels after the recovery period (Fig. 3). In most cases, however, the results were not significant because of the high inter-individual variation in the biochemical response (Fig. 3). The ATP concentration and the overall adenylate pool did not differ significantly between exercise groups in smaller or bigger individuals throughout the swimming experiment, but were overall lower in bigger (older) than young animals (Fig. 4A,B; P<0.05).

Glycogen concentrations were significantly higher in smaller than in bigger individuals of groups 0 and 4 (Fig. 5). Furthermore, glycogen concentrations decreased significantly in smaller individuals throughout the swimming experiment

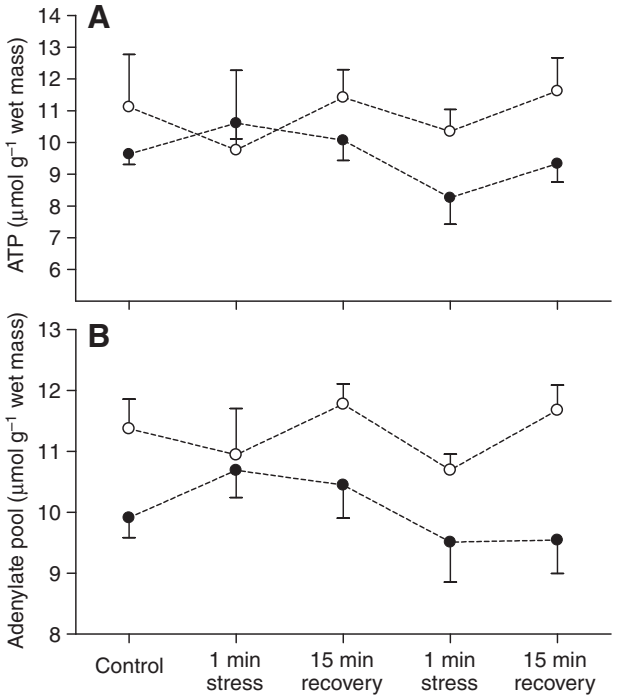

Fig. 4. Concentration of (A) ATP and (B) adenylate pool in adductor muscle of different experimental groups of smaller (open circles, *N*=4–10) and bigger (filled circles, *N*=8–12) individuals. No significant differences between groups were found but overall ATP concentration and adenylate pool of bigger individuals was significantly lower than those of smaller individuals. JMP statistic two-way ANOVA $P<0.05$ for age and >0.05 for group and age × group effect.

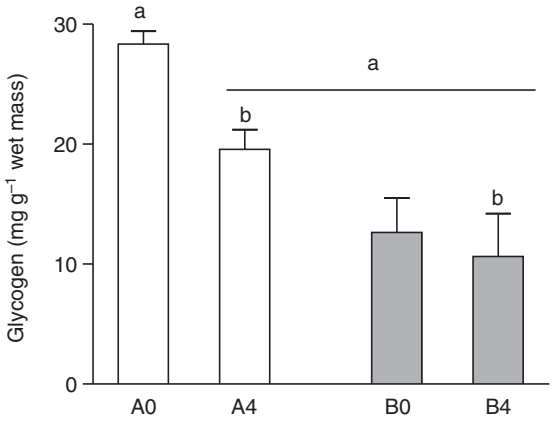

Fig. 5. Glycogen concentration in *A. opercularis* muscle tissue of unstressed animals (group 0) and animals that underwent complete run 1 and run 2 (group 4). White bars: smaller individuals [A0, A4: *N*=8, 11; shell height 50.97±4.473 mm (mean ± s.d.)]; grey bars: bigger individuals (B0, B4; *N*=11, 15; shell height 69.30±2.611 mm). Bars with the same letter are significantly different from each other (Student's *t*-test, $P<0.05$).

whereas in bigger individuals no change in glycogen content was observed (Fig. 5). Total glutathione (tGSH) and reduced glutathione (GSH) concentrations in muscle tissue of *A. opercularis* were overall significantly higher in the smaller compared with the bigger individuals and differed between groups (two-way ANOVA; Fig. 6A,B). The swimming experiment affected glutathione concentrations in smaller and bigger individuals in different ways (Fig. 6). Smaller individuals suffered a significant decrease in tGSH and GSH during the swimming experiment whereas in bigger individuals a trend was seen in both parameters but did not reach significance (Fig. 6A,B).

Oxidized glutathione (GSSG) concentrations were very low (Table 3) and no significant change occurred in muscle tissue of either smaller or bigger *A. opercularis* during the swimming experiment (Table 3). Thus the change in the total glutathione concentration (Fig. 6A) mainly results from the change in the reduced glutathione fraction (Fig. 6B). Owing to the very low GSSG concentration no significant change in the GSSG:GSH ratio was observed in smaller or bigger individuals during the swimming experiment and the ratios did not differ between age/size groups (Table 3). Adductor muscle pH decreased significantly throughout the swimming experiment in both size groups (Fig. 7). Once again a difference in cellular stress response was found between smaller and bigger individuals. In smaller individuals, the tissue pH decreased in both recovery periods, whereas in the bigger individuals the muscle pH stabilized and did not decrease further in the second recovery period. Measurements of tissue pH were carried out in the same muscle sample used for the glutathione measurements in order to calculate cellular redox potential [after Schafer and Büttner (Schafer and Büttner, 2001)]. Redox potential shifted to more oxidized values throughout the swimming experiment in smaller and bigger individuals (Fig. 8). However, again changes between control and second recovery phase were only significant in the small animals. The antioxidant enzyme catalase was only measured in unstressed control animals (group 0). Catalase activities were not significantly different between smaller and bigger individuals of group 0 (Table 3).

Lipidperoxidation under exercise and recovery was measured in the form of lipid hydroperoxides (CHPE) and malondialdehyde. Both parameters did not change in muscle tissue of either smaller or bigger individuals during the swimming experiment (Table 3). However, lipid hydroperoxides were overall lower in the bigger than the smaller individuals (Table 3).

DISCUSSION

Smaller and bigger *A. opercularis* differ markedly in their physiological response during exercise, corresponding to major changes of adductor muscle cellular composition and physiology as determined in this study of ageing. Age was determined from size, based on a previously obtained von Bertalanffy curve and although not as precise as counting yearly growth rings of individual shells, does result in reliable age data, because in *A. opercularis* age and size are significantly correlated. We can therefore assume that the two size classes given in the present paper represent different age clusters.

Adductor muscle of older individuals had lower mitochondrial volume densities, less specific CS activity, lower ATP and generally lower adenylate concentrations compared with muscle tissue of younger individuals. Moreover, the energetic reserve glycogen was less concentrated in older animal adductor muscle. This is in line with data of Tremblay et al. (Tremblay et al., 2006), who found a decrease in arginine kinase (AK) activity, the enzyme responsible for the restoration of ATP *via* phospho-arginine with increasing size of the scallop *Chlamys islandica* (size range 24–35 mm) from Eastern Canada. In contrast to an earlier study of the same queen scallop population (Philipp et al., 2006), that did, however, involve a larger size and age range of animals, in the present investigation we found only an insignificant decline in respiration rates of

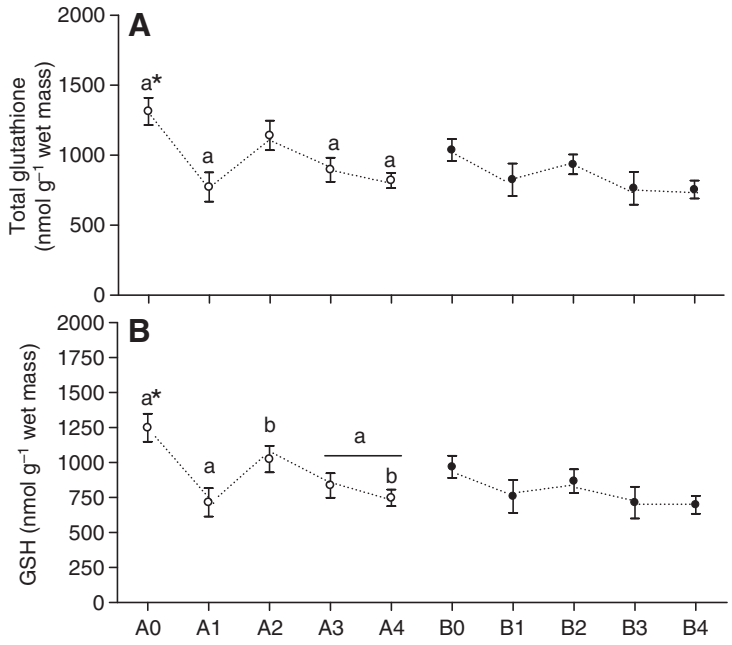

Fig. 6. Total glutathione [2 oxidised (GSSG) + reduced (GSH); A] and reduced glutathione (B) concentration in smaller [A0–A4, open circles; shell height 50.45±4.04 mm (mean ± s.d.)] and bigger [B0–B4. filled circles; shell height 68.18±2.75 mm (mean ± s.d.)] *A. opercularis* individuals of group 0–4. *N*=9–17 per group for smaller and *N*=9–18 per group for bigger individuals. Groups with the same letter (small letters for smaller) are significantly different from each other ($P<0.001$). If more than two groups have the same letter, the group marked with an asterisk is significantly different to all other groups.

mitochondria isolated from the older animals. The age-related decrease in aerobic scope still became visible in the reduction of mitochondrial volume density and citric acid cycle enzyme activity, as well as in lower ATP and adenylate concentrations of older compared to young individuals. Thus older individuals appear to suffer a reduction of both the aerobic and the anaerobic energetic capacity in their swimming muscle.

An initial part of this study (Schmidt et al., 2008), covering behaviour and morphometrics of differently sized *A. opercularis*, revealed that older scallops additionally have smaller adductor muscles relative to their heavier shells, and that the water drag the animals experience during swimming is higher in bigger scallops, indicating the energetic expenditures for swimming could be higher in the older animals. Yet surprisingly, counting the number of claps on predator attack we found no difference in the swimming activity between young and older individuals. Thus lower energetic capacity in the older animals does not apparently impair their ability to swim and escape a predator. This is in keeping with a similar change in adenylates and energy charge in young and old individuals, and swimming thus seems to be equally consuming for both age groups. However, marked differences in shell closing behaviour between younger and older individuals were found with 47% of younger *A. opercularis* closing their shell during predator attack and keeping it closed for at least another 30 min, whereas such prolonged shell closure was observed in only 3% of the older experimental animals. Scallop species differ with respect to behaviour after exhaustive swimming: *Argopecten iridans concentricus* (Chih and Ellington, 1983) shows a wider shell gape, probably to accelerate water exchange, whereas *Placopecten magellanicus* (Thompson et al., 1980) were found to close their shells tightly and to take several hours before opening up again. Wider shell gape after exhaustive swimming was also observed in the present study with *A. opercularis*

Table 3. Malondialdehyde, lipid hydroperoxides and oxidized glutathione concentration, ratio of oxidized and reduced (GSH) glutathione and catalase activity in adductor muscle of smaller and bigger *A. opercularis* individuals of the different experimental groups

	Size (mm)	Control	1 min stress	15 min recovery	1 min stress	15 min recovery	*Significance
MDA (μmol g^{-1} wet mass)	Smaller 50.00±3.03	53.73±3.498	62.38±4.429	55.27±6.981	56.85±7.671	54.71±3.006	n.s.
MDA (μmol g^{-1} wet mass)	Bigger 68.83±2.71	54.25±3.49	73.75±15.43	41.02±3.333	50.37±5.009	44.93±3.724	n.s.
CHPE (nmol g^{-1} wet mass)	Smaller 50.70±3.56	986.2±58.39	903.7±107.3	942.2±53.25	1010±97.27	1006±62.4	*
CHPE (nmol g^{-1} wet mass)	Bigger 68.67±2.75	891.9±66.26	933±67.31	794.2±99.42	822.2±85.93	734,7±47.92	*
GSSG (nmol g^{-1} wet mass)	Smaller 50.45±4.04	32.49±4.111	30.15±5.27	28.13±2.432	29.52±2.661	35.9±4.432	n.s.
GSSG (nmol g^{-1} wet mass)	Bigger 68.18±2.75	33.98±5.789	32.9±6.683	24.75±2.982	25.5±2.689	27.55±2.776	n.s.
GSSG/GSH	Smaller 50.45±4.04	0.028±0.005	0.044±0.008	0.026±0.001	0.042±0.009	0.060±0.012	n.s.
GSSG/GSH	Bigger 68.18±2.75	0,038±0.0082	0.0586±0.017	0.028±0.003	0.04±0.008	0.045±0.005	n.s.
Catalase† (i.u. g^{-1} wet mass)	Smaller 50.45±4.04	128.4±11.25	n.d.	n.d.	n.d.	n.d.	n.s.
Catalase† (i.u. g^{-1} wet mass)	Bigger 68.18±2.75	115.1±8.72	n.d.	n.d.	n.d.	n.d.	n.s.

MDA, malondialdehyde (*N*=4–11); CHPE, lipid hydroperoxides (*N*=5–12); GSSG, oxidized glutathione (*N*=8–18); GSSG, oxidized glutathione; GSH, reduced glutathione (*N*=8–18) and catalase (*N*=9–11).

Data are means ± s.e.m. (or means ± s.d. for size).

†Catalase was only measured in group 0 animals. n.d., not determined; *significantly different ($P<0.05$) between size/age groups; n.s., not significantly different between size/age groups.

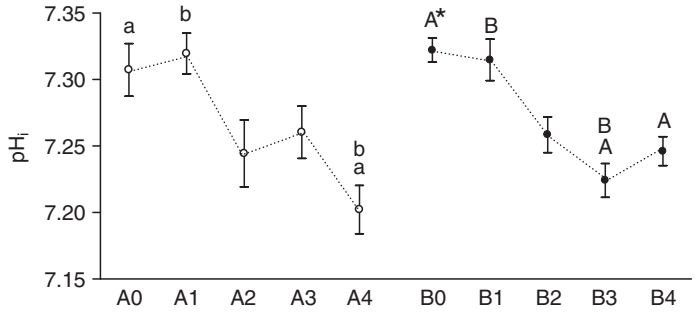

Fig. 7. Tissue pH (pH_i) in muscle tissue of different *A. opercularis* experimental groups. A0–A4 smaller individuals [open circles, *N*=8–16, shell height 50.34±4.085 mm (mean ± s.d.)], B0–B4: bigger individuals [filled circles, *N*=9–18; shell height 68.84±2.91 mm (mean ± s.d.)]. Groups with the same letter (smaller individuals=small letters, bigger individuals=capital letters) are significantly different from each other (*P*<0.001). When more than two groups have the same letter, the group marked with an asterisk is significantly different from the other two groups.

individuals (personal observation) mainly in older specimens that stayed open, whereas nearly 50% of the younger individuals closed after swimming.

Interestingly, in their study of *Chlamys islandica*, Tremblay et al. (Tremblay et al., 2006) found decreased arginine kinase and CS activities with age, thus confirming the decline in aerobic capacity in old scallops, as well as elevated pyruvate kinase (PK), octopine dehydrogenase (ODH) and glycogen phosphorylase (GP) activities in larger animals, indicating bigger/older C*hlamys islandica* to be better equipped for anaerobic energy production than the smaller conspecifics.

The difference in swimming energetics mainly in the older *A. opercularis*, which had lower glycogen concentrations than younger individuals, and the fact that older animals neither mobilized glycogen reserves during exhaustive swimming and recovery nor closed their shell after exhaustion, indicates, however, that anaerobic glycolysis might be of minor significance in older queen scallops and suggests that they rely mainly on aerobic energy production. The results of the muscle pH measurements further corroborate this hypothesis: in molluscs, octopine was long regarded as a preferable metabolic end product over lactate to prevent tissue acidification during exercise. Later it was established that metabolic acidosis could be caused also by octopine in squid (Pörtner et al., 1996; Pörtner et al., 1991) as octopine generation, like lactate formation, leads to proton release and acidification (Hochachka and Mommsen, 1983). In *A. opercularis* (Grieshaber, 1978) and some other scallop species (Chih and Ellington, 1983; Livingstone et al., 1981) octopine generation takes place only during recovery whereas swimming is powered by transphosphorylation of arginine phosphate. In the present study, pH_i decreased in both age groups in the first recovery period, which indeed points to an acidifying effect of octopine formation. In younger individuals, muscle pH decreased further in the second recovery phase indicating that more octopine was generated, whereas levels remained stable in older individuals. In maintaining their shell open, the older queen scallops may have been able to prevent the stronger acidification and enabled aerobic energy recuperation and octopine degradation.

In summary, the older animals employ a different strategy to maintain the energetic balance in adductor/swimming muscle on repeated predator attack. Although they seem to have less powerful muscles, that are smaller relative to their big and heavy shells and with less mitochondrial volume density, enzyme activities and adenylate concentrations, they manage to prevent strong acidification and accumulation of anaerobic octopine by keeping their shell open during recovery and supporting aerobic replenishment of energy reserves. This reduces the need to catabolize octopine, which consumes additional ATP (Livingstone et al., 1981) and, moreover, presumably prevents a significant drop in the Gibbs free energy of ATP hydrolysis which occurs in tissues at more acidic pH (Zielinski and Pörtner, 1996). The more stable glycogen concentration, pH_i values and cellular redox state throughout the swimming experiment in older compared with younger individuals corroborate this hypothesis.

Glutathione metabolism in exercising queen scallops

In line with several studies investigating thiol concentrations during exercise in different tissues and blood (Medved et al., 2004), total (tGSH) and reduced glutathione (GSH) concentrations decreased significantly during the swimming experiment, whereas the oxidised glutathione (GSSG) concentration remained constant. In humans, Medved et al. (Medved et al., 2004) found GSSG levels to increase in the blood of exercising subjects, whereas total glutathione levels remained constant, indicating release of GSSG from exercising tissue. Organisms avoid high cellular GSSG concentrations because the oxidized glutathione has toxic effects through protein glutathionylation [protein-SH + GSH = protein-SSG (Han et al., 2006)] and oxidation of the cellular redox potential. Thus, GSSG is generally transported out of the cells using energy consuming transporters (Ishikawa et al., 1986; Leier et al., 1996; Sen and Packer, 2000). In the present study, the glutathione oxidized

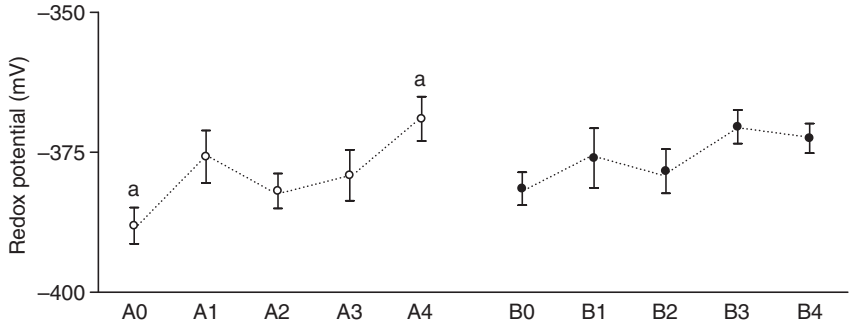

Fig. 8. Cellular redox potential in muscle tissue of *A. opercularis* individuals throughout the swimming experiment. A0–A4 smaller individuals [open circles, *N*=8–17, shell height 50.42±4.185 mm (mean ± s.d.)]; B0–B4: bigger individuals [filled circles, *N*=9–18, shell height 69.18±2.747 mm (mean ± s.d.)]. Groups with the same letter are significantly different from each other with *P*<0.05.

during exhaust swimming (GSSG) is likely to have been released from the muscle tissue into the haemolymph of the exercising scallops in order to prevent glutathionylation of adductor muscle proteins. Mantle tissue and adductor muscle are well perfused in scallops (Drew, 1907) and swimming additionally accelerates haemolymph circulation (Drew, 1907) and could support removal of oxidised glutathione from the tissue.

The more pronounced decrease in glutathione in younger individuals again reflects the difference in swimming physiology between animal size/age groups. Furthermore, the calculation of the redox potential after Schafer and Büttner (Schafer and Büttner, 2001) using GSH, GSSG and muscle pH shows that the difference in escape response also affects the cellular redox ratio.

Lipidperoxidation in ageing, growth and exercise

Unspecific oxidation of lipids 'lipidperoxidation' commences with lipid radical production and leads to the accumulation of stable degradation products, such as malondialdehyde (Sies, 1986). During exercise elevated metabolic rates can lead to higher ROS generation and thus higher lipidperoxidation. However, as suggested by McArdle and Jackson in recent reviews (Jackson, 2005; McArdle and Jackson, 2000) and stated above, ROS generation during exercise does not necessarily lead to oxidative damage as it might be tightly controlled by antioxidants and protecting proteins such as heat shock proteins. In the present study we could not find a significant increase in two parameters measuring lipidperoxidation during the swimming experiment. This might be due to the ROS scavenging capacity of the high glutathione concentrations found in the adductor muscle when compared to mantle tissue, as found for this species and the temperate mud clam *Mya arenaria* (Philipp et al., 2005a; Philipp et al., 2006), preventing lipidperoxidation. Catalase activities were similar in younger and older individuals, whereas glutathione concentrations decreased with age in *A. opercularis*. Compared with mantle tissues of *A. opercularis*, catalase activities were quite low in muscle tissue, whereas glutathione concentrations were double those of the mantle tissue of *A. opercularis* (Philipp et al., 2006) as well as of North Sea mud clams (Philipp et al., 2005a; Weihe, 2005). Lower catalase activities in the striated muscle of scallops compared to the non-striated portion and to adductor muscles of less active mud clams seem to be a general finding (Hopkins, 1934), which reflects the ROS buffering function of glutathione rather than catalase during exercise. Sen and Packer et al. (Sen and Packer et al., 2000) even extended the importance of glutathione in exercise to all cellular thiols. The assays used for lipidperoxidation are widely used but under discussion for specificity and reliability. Other methods, e.g. MDA measurement by HPLC might give more detailed results concerning lipidperoxidation under exercise. However, the similar results for lipidperoxidation obtained by the two different methods corroborates the assumption that no lipidperoxidation occurred under exercise in the present study.

Altogether *A. opercularis* seems to be well equipped with an antioxidant defence that is in line with its active lifestyle. Compared with other bivalves (Abele, 2002; Heise et al., 2003; Philipp et al., 2005b) ROS generation rates of isolated mitochondria from adductor muscle tissue (present study) and mantle tissue (Philipp et al., 2006) are extremely low, which may be an adaptation for the active lifestyle of the scallops, to prevent extensive ROS generation in frequent phases of exhaustive exercise. This is in agreement with earlier studies on ageing of polar and temperate mud clams and scallops (Philipp et al., 2005a; Philipp et al., 2006).

In summary, young and older animals showed differences in physiology before and during predator escape. Older individuals were found to have lower aerobic energetic capacity (mitochondrial volume density and enzyme activity and adenylates) as well as anaerobic energy generation capacity, but cellular changes in redox potential, pH and loss of glutathione in the swimming muscle was more pronounced in younger queen scallops, thus older individuals more effectively stabilized cellular homeostasis during repeated exercise than did the younger individuals.

An earlier study showed that swimming itself is not impaired by the biochemical changes in the young animals (Schmidt et al., 2008). Valve closure frequency is less in older than younger individuals, on the one hand supporting maintenance of cellular homeostasis during swimming and recovery in older scallops, and on the other hand it may be a consequence of the lower capacity for anaerobic energy generation in the old animals.

A decrease in swimming capacity thus may not be the direct cause for the short maximum life span of 8–10 years (Ansell et al., 1991; Philipp et al., 2006) in *A. opercularis*, however, the loss of shell closure ability might increase the likelihood of predation in aged specimens. What factors eventually result in the short life expectancy and let the animals die at such a young age remains speculative and needs to be investigated. Possibly, structural and functional deterioration will manifest only in animals closer to the maximum reachable age in a species, animals, which in case of the queen scallop, fall prey to commercial fishing.

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