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RESEARCH ARTICLE

Mitochondrial performance of a continually growing marine bivalve, *Mytilus edulis*, depends on body size

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ABSTRACT

Allometric decline of mass-specific metabolic rate with increasing body size in organisms is a well-documented phenomenon. Despite a long history of research, the mechanistic causes of metabolic scaling with body size remain under debate. Some hypotheses suggest that intrinsic factors such as allometry of cellular and mitochondrial metabolism may contribute to the organismal-level metabolic scaling. The aim of our present study was to determine the metabolic allometry at the mitochondrial level using a continually growing marine ectotherm, the mussel *Mytilus edulis*, as a model. Mussels from a single cohort that considerably differed in body size were selected, implying faster growth in the larger specimens. We determined the body mass-dependent scaling of the mitochondrial proton leak respiration, respiration in the presence of ADP indicative of the oxidative phosphorylation (OXPHOS), and maximum activity of the mitochondrial electron transport system (ETS) and cytochrome *c* oxidase (COX). Respiration was measured at normal (15°C), and elevated (27°C) temperatures. The results demonstrated a pronounced allometric increase in both proton leak respiration and OXPHOS activity of mussel mitochondria. Mussels with faster growth (larger body size) showed an increase in OXPHOS rate, proton leak respiration rate, and ETS and COX activity (indicating an overall improved mitochondrial performance) and higher respiratory control ratio (indicating better mitochondrial coupling and potentially lower costs of mitochondrial maintenance at the same OXPHOS capacity) compared with slower growing (smaller) individuals. Our data show that the metabolic allometry at the organismal level cannot be directly explained by mitochondrial functioning.

KEY WORDS: Mitochondrial respiration, Allometric scaling, Metabolic rate, Proton leak, Temperature, Growth rate

INTRODUCTION

Metabolic rate is a fundamental biological rate that defines the physiological and life-history performance and has direct implications for Darwinian fitness and the ecological role of an organism (Brown et al., 2004; da Silva et al., 2006; Calosi et al., 2013). The allometric body mass scaling of metabolic rate is one of the longest recognized and best documented phenomena in animals (Kleiber, 1932; see also J. Exp. Biol. special issue, 'Scaling Functions to Body Size: Theories and Facts' – Hoppeler and Weibel, 2005). Mass-specific metabolic rate declines with increasing body size, so that larger organisms have lower metabolic turnover and energy demand per unit mass than smaller-bodied organisms. This pattern is commonly found across different animal taxa, among organisms of different sizes within a single species (review by Konarzewski and Książek, 2013) and within a single individual during ontogenesis (Moses et al., 2008; Maino and Kearney, 2014). Commonly, the allometric relationship between metabolic rate (R) and body mass (M) is expressed in the form of a power function: $R=aM^b$, where b is the metabolic scaling coefficient and a is a proportionality constant. The value of the metabolic scaling coefficient (0.75 or ¾) originally calculated by Kleiber (1932) has been proposed as a universal scaling constant from molecules to whole organisms and ecosystems (West et al., 2002; Brown et al., 2004) but the universality of the scaling coefficient has been extensively criticized on both empirical and theoretical grounds (Darveau et al., 2002; Carey et al., 2013; Hulbert, 2014; Glazier, 2015). Notwithstanding the debate about the universality and the value of the metabolic scaling coefficient, the fact that most animals, irrespective of their physiology, habitat or lifestyle, conform to the pattern of declining mass-specific metabolic rate with increasing body size remains uncontroversial and implies the existence of some metabolic constraints underlying this pattern (Darveau et al., 2002; Hulbert, 2014; Ballesteros et al., 2018; Thommen et al., 2019).

One of the important open questions in the metabolic theory of ecology is the mechanistic underpinning of body mass-dependent metabolic scaling (Hulbert, 2014; Maino et al., 2014; Ballesteros et al., 2018). Multiple hypotheses have been proposed to explain these mechanisms with system-level constraints such as the size-dependent limitations of the oxygen and nutrient transport (West et al., 2002), body-mass dependent changes in the relative proportion of organs and tissues with different metabolic demands (Darveau et al., 2002; Suarez and Darveau, 2005), ontogenetic shifts in the energy investment in processes such as heat production, locomotion, growth and reproduction (Glazier, 2005), changes in the structure and function of cellular and mitochondrial membranes affecting the cellular energy-dependent transport processes and thus rate of metabolism (Hulbert and Else, 1999), or negative genetic correlations between growth rate and metabolism (White et al., 2019). It has also been proposed that intrinsic factors such as allometry of cellular and mitochondrial metabolism may contribute to organismal-level metabolic scaling (Porter and Brand, 1995a,b; Porter et al., 1996; Porter, 2001; Glazier, 2015). Several studies demonstrated allometric decline in metabolic rate at sub-organismal (tissue and cellular) levels with increasing body size (Porter and Brand, 1995b; Savina et al., 1997; Hulbert et al., 2002; West et al., 2002; West and Brown, 2005), while others did not find such a relationship (Burpee et al.,

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Received 6 April 2020; Accepted 2 June 2020

2010; Sukhotin et al., 2017; Boël et al., 2020). Furthermore, the issue is complicated by the potentially confounding effects of phylogeny in interspecific comparisons, and by the correlation between age and size in intraspecific analyses of metabolic scaling.

In aerobic organisms, mitochondria are responsible for >90% of ATP production that occurs via the chemiosmotic coupling of electron transport and ATP synthesis in the process of oxidative phosphorylation (OXPHOS). If intrinsic factors (such as cellular and mitochondrial respiration) are contributing to metabolic scaling, the allometric scaling for mitochondrial metabolism would be expected to reflect the pattern of aerobic metabolism in cells, tissues and organisms. To date, most studies on the allometry of cellular and mitochondrial respiration have been performed on endotherms or vertebrate ectotherms and reported the independence of mitochondrial respiration from body mass (e.g. in mammals: Taylor, 1987, Porter and Brand, 1993; Porter, 2001; in fish: Burpee et al., 2010; and in amphibians: Roussel et al., 2015). Allometry of cellular respiration (if observed) was determined by the number, volume or membrane surface area of the mitochondria or by the rate of ATP turnover rather than by mitochondrial functional capacity (Else and Hulbert, 1985a,b; Porter and Brand, 1995a,b; Porter et al., 1996; Porter, 2001). In a notable exception, mitochondrial respiration [reflecting electron transport system (ETS) activity needed to compensate for all futile proton and cation cycles that dissipate the mitochondrial membrane potential without generating ATP] decreased with increasing body size across different species of mammals (Porter and Brand, 1995a,b; Porter et al., 1996; Porter, 2001), possibly reflecting adjustments of metabolic heat generation to compensate for body size-dependent heat dissipation (Ballesteros et al., 2018).

Recent research showed a functional link between intraspecific variation in mitochondrial performance and individual growth rates in several species of vertebrates including endotherms such as rats, pigs (Lutz, 2003), chickens and cows (Bottje and Carstens, 2009), and ectotherms such as frogs (Salin et al., 2012a,b). Cross-species comparisons in 12 species of mammals showed that mitochondrial efficiency (measured as ATP:O ratio) was positively correlated with body mass when mitochondria were functioning at close to the basal metabolic rate; however, the efficiency was independent of body mass in mitochondria respiring at the maximum OXPHOS rate (Mélanie et al., 2019). Higher mitochondrial efficiency implies better coupling (and therefore lower rates of proton leak and/or electron slip in the mitochondria of the faster growing animals) and predicts different allometries for the OXPHOS and proton leak rates. Most studies on cellular and mitochondrial metabolic scaling were carried out on endothermic animals, in which thermoregulation strongly affects mitochondrial performance. Studies investigating the relationship between mitochondrial activity and body size (or growth rate) are scarce in ectotherms and require further investigation.

The aim of our present study was to determine the metabolic allometry at the mitochondrial level using a continually growing marine ectotherm, the blue mussel *Mytilus edulis*, as a model. Blue mussels, *Mytilus* spp., are common species in the intertidal and shallow subtidal zones around the world and, like many bivalve molluscs, are characterized by great individual variability of growth rate (Sukhotin et al., 2007). Furthermore, mussels are a long-lived species (maximum longevity of 7–24 years depending on the population) (Sukhotin et al., 2007). This allows for separation of the potential effects of age and body size when investigating metabolic scaling in mussels. We selected mussels from a single cohort (3–4 years old) that considerably differed in body size, implying faster growth in the larger specimens. We hypothesized that if mitochondrial metabolic allometry is an important intrinsic factor contributing to organismal metabolic scaling (Glazier, 2015), the overall mitochondrial respiration (including OXPHOS and proton leak) will decline with increasing body mass in mussels. However, if mitochondrial efficiency rather than overall mitochondrial respiration is an important contributor to whole-animal metabolic allometry, we expect a stronger body mass-dependent decrease in mitochondrial proton leak than in OXPHOS or ETS capacity with increasing body mass. To test these hypotheses, we determined the body mass-dependent scaling of mitochondrial proton leak respiration, OXPHOS, and maximum activity of the mitochondrial ETS and the terminal cytochrome *c* oxidase (COX) in mussel mitochondria. We also determined whether a change in overall mitochondrial activity (induced by a temperature upshift from 15°C to 27°C) alters the allometry of mitochondrial metabolism in this model marine ectotherm.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Thermo Fisher Scientific (Schwerte, Germany), and were of analytical grade or higher.

Animal collection and maintenance

Blue mussels *Mytilus edulis* Linnaeus 1758 were collected from the mussel culture farm located near the White Sea Biological Station Kartesh in Chupa Inlet (66°20′14.27″N, 33°38′12.10″E, Kandalaksha Bay, White Sea) in August 2017 (seawater temperature +15°C, salinity 24 practical salinity units, psu). Only specimens 3–4 years old were selected for experiments. Molluscs were taken from substrates exposed for 3 and 4 years, and the age of animals was confirmed by analysis of the growth marks on the shells. Specimens were selected to cover the widest possible size range from 1.7 to 5.7 g wet tissue mass. Mussels were transported to the Department of Marine Biology, University of Rostock (Germany) and placed in aquaria (artificial seawater, 25 psu, +15°C) for 7 days prior to the experiments. Mussels were fed *ad libitum* on alternate days by addition of a commercial algal blend (DT's Live Marine Phytoplankton, Sycamore, IL, USA) according to the manufacturer's instructions.

Mitochondrial isolation and high-resolution respirometry

Mitochondria were isolated from the digestive gland of the mussels as described elsewhere (Falfushynska et al., 2019). Briefly, ∼1 g of tissue (pooled from 2–4 mussels, depending on their size) was homogenized in ice-cold homogenization media containing 100 mmol l^{-1} sucrose, 200 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl, 8 mmol l^{-1} EGTA, 50 µg l^{-1} aprotinin, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and 30 mmol l^{-1} Hepes, pH 7.5, using several passes of a Potter–Elvenhjem homogenizer at 200 rpm. Within each pooled group, mussels of similar size (within 25% of body mass) were used. Mitochondria were isolated by differential centrifugation at 4°C, first for 4 min at 2000 ***g*** to remove cell debris, and then for 8 min at 8000 ***g*** to collect the mitochondria. The mitochondrial pellet was resuspended in ice-cold assay media containing 440 mmol l^{-1} sucrose, 130 mmol l^{-1} KCl, 10 mmol l^{-1} NaCl, 30 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KH_2PO_4 and 1% bovine serum albumin (BSA), pH 7.2. In total, 22 independent preparations were made.

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

Respiratory state or index	Reflective of:	Determined as:
PL	Proton leak	Respiration in the presence of oligomycin
OXPHOS	OXPHOS capacity	Respiration in the presence of ADP
ETS	ETS capacity	Respiration after the addition of an uncoupler (CCCP)
COX	Cytochrome *c* oxidase capacity	Respiration after addition 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

Oxygen consumption of isolated mitochondria was measured using an Oxygraph 2k high-resolution respirometer (Oroboros, Innsbruck, Austria) at 15 and 27°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, 2 mmol l^{-1} malate and 10 mmol l^{-1} succinate to fully stimulate the electron flux at Complexes I and II of the ETS; 2.5 mmol l^{-1} ADP to achieve OXPHOS (ADP stimulated) respiration; 2.5 µmol l^{-1} oligomycin to inhibit mitochondrial F_O, F_1-ATPase and achieve resting respiration indicative of proton leak; 7.5 µmol 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 COX; and 20 mmol l^{-1} KCN to inhibit COX. Remaining mitochondrial isolates were lysed by several freeze–thaw cycles in hypoosmotic solution, and protein concentration was measured at 595 nm using a Bradford Protein Assay Kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). BSA was used as a standard. Mitochondrial respiration was expressed as µmol O_2 min^{-1} g^{-1} mitochondrial protein.

Calculations and statistics

Mitochondrial respiratory control ratio (RCR) and relative OXPHOS, ETS and COX flux (Table 1) were calculated as described elsewhere (Estabrook, 1967; Gnaiger, 2012). The temperature dependence of different mitochondrial activities was expressed as Q_{10} (Cossins and Bowler, 1987). Data were tested for normality and homogeneity of variances using Kolmogorov–Smirnov and Levine tests, respectively. The effects of temperature and body size of mussels on mitochondrial performance were tested by ANCOVA, with temperature as a fixed factor and body mass as a covariate. To assess the allometry of different metabolic activities, power regressions were fitted to the data in Excel (Microsoft Office 2016) with the respective mitochondrial activity as a dependent variable and body mass of the mussel as an explanatory variable. The R^2 was used to assess the goodness-of-fit for all regressions. Effects were considered significant if the probability of Type I error was less than 0.05. All data (n=22) are reported as the mean±s.e.m. unless indicated otherwise.

RESULTS

Effect of temperature

As expected, elevated temperature accelerated mussel mitochondrial activity (Fig. 1, Table 2). The sensitivity to warming differed between different mitochondrial functions. Mitochondrial proton leak respiration increased with temperature to a lesser degree (Q_{10}=1.9) than the rates of OXPHOS and ETS (Q_{10}=2.6–2.9) (Fig. 1A–C). The RCR, which shows the excess of OXPHOS respiration over proton leak, was lower at 15°C than at 27°C (1.94±0.15 versus 3.07±0.18, respectively) (Fig. 1E).

A temperature-induced increase in the activity of the terminal ETS oxidase, COX, was significant (P<0.001) but less pronounced (Q_{10}=1.65–1.95, depending on the mussels' size) than for the entire ETS (Q_{10}=2.6–2.8). The difference in response to elevated temperature of OXPHOS and COX activity determined a strong negative effect of warming on COX/OXPHOS ratio (Fig. 1G). The ratio of ETS to OXPHOS activity did not change with temperature (1.5 and 1.7 at 15°C and 27°C, respectively; Fig. 1F).

Effect of body size

Mitochondrial oxygen consumption was significantly body mass dependent in *M. edulis* (Table 2), albeit the strength of the allometric effect varied between different mitochondrial activities. OXPHOS and COX activity showed the strongest body mass dependence, with an almost linear increase in oxygen flux with body mass (reflected in the metabolic scaling coefficient b≈1) (Fig. 2B,D). Mitochondrial proton leak respiration accelerated less rapidly with body mass (b=0.657–0.662) (Fig. 2A). Similar metabolic scaling coefficients were found for uncoupled mitochondrial respiration, reflecting maximum ETS activity (b=0.563–0.600) (Fig. 2C). Overall, body mass explained 28–30% of the total variation in mitochondrial proton leak respiration and 33–43% of the variation in OXPHOS rates. Elevated temperature (27°C) had no effect on the metabolic scaling of the studied mitochondrial rates.

RCR slightly increased with mussel body mass (Fig. 3A), but the relationship was weak (b=0.277–0.364), with body mass explaining 9–11% of the RCR variation (P=0.07) (Table 2). Reserve ETS capacity (measured as the ratio of ETS to OXPHOS activity) showed a pronounced decline with body size of molluscs (Fig. 3B). In the smallest mussels, this ratio was >2, while in the biggest studied specimens it was close to 1, indicating that OXPHOS flux was approaching the limit set by the ETS flux capacity (Fig. 3B).

Table 2. ANCOVA: Effects of temperature and body size on bioenergetics-related traits of isolated mitochondria from the digestive gland of *M. edulis*

	Body mass		Temperature	
Trait	*F*	*P*	*F*	*P*
PL	**13.39**	**<0.001**	**79.08**	**<0.001**
OXPHOS	**17.27**	**<0.001**	**79.36**	**<0.001**
ETS capacity	**15.13**	**<0.001**	**153.08**	**<0.001**
COX	**19.18**	**<0.001**	**26.71**	**<0.001**
RCR	3.38	0.073	**22.72**	**<0.01**
COX/ETS	0.00004	0.995	**52.07**	**<0.001**
ETS/OXPHOS	**7.15**	**<0.05**	1.47	0.233

There is one degree of freedom for the factor temperature, one degree of freedom for the covariate (body mass), and 41 degrees of freedom for the error. Trait abbreviations are given in Table 1. Significant effects are in bold.

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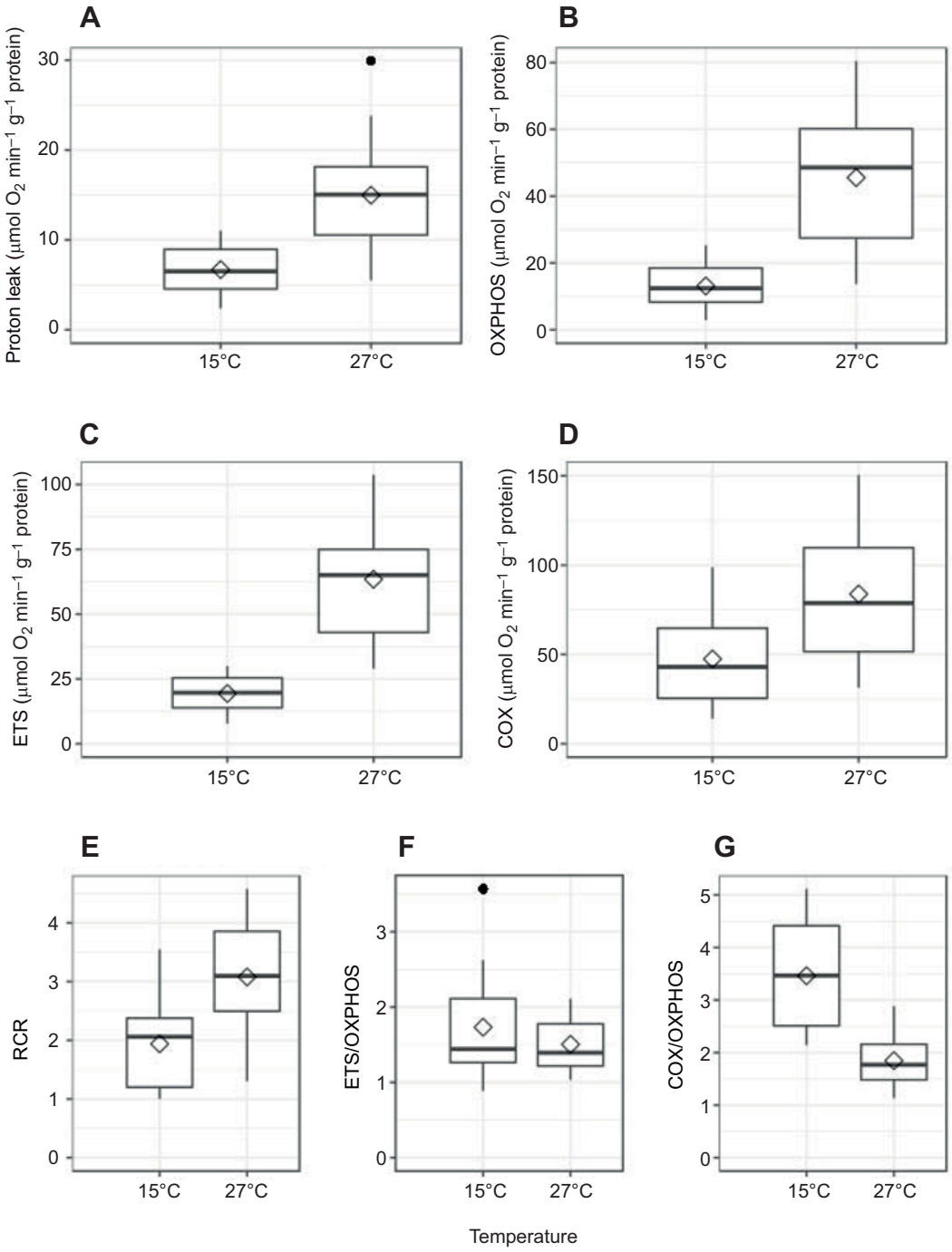

Fig. 1. Mitochondrial performance in digestive gland of mussels at normal (15°C) and elevated (27°C) temperature. (A) Proton leak respiration. (B) Active respiration indicative of oxidative phosphorylation (OXPHOS). (C) Maximum activity of the complete mitochondrial electron transport system (ETS). (D) Maximum activity of cytochrome *c* oxidase (COX). (E) Respiratory control ratio (RCR). (F) Reserve ETS capacity. (G) Reserve COX capacity. Mean (diamonds) and median (horizontal line inside boxes) values, upper and lower quartiles (box), 1.5× interquartile range (whiskers) and outliers (dots) are presented.

COX reserve capacity (i.e. the ratio of COX to OXPHOS activity) was body mass independent and decreased with increasing temperature from >3 at 15°C to ∼2 at 27°C (Fig. 3C).

DISCUSSION

Metabolic allometry at different levels of biological organization

The decline in mass-specific metabolic rate with increasing size in animals has been documented for over 100 years, yet the mechanisms underlying this pattern remain debated (Kleiber, 1932; Niklas and Kutschera, 2015). Organismal metabolic rate is composed of the metabolic rates of tissues and organs, which are in turn determined by cellular energetics and mitochondrial functions (e.g. Suarez et al., 2004). Therefore, whole-animal metabolic allometry could be reflected in (or determined by) the similar patterns of metabolic processes in suborganismal structures. Indeed, decline in cellular and mitochondrial respiration with increasing body mass has been recorded in interspecific

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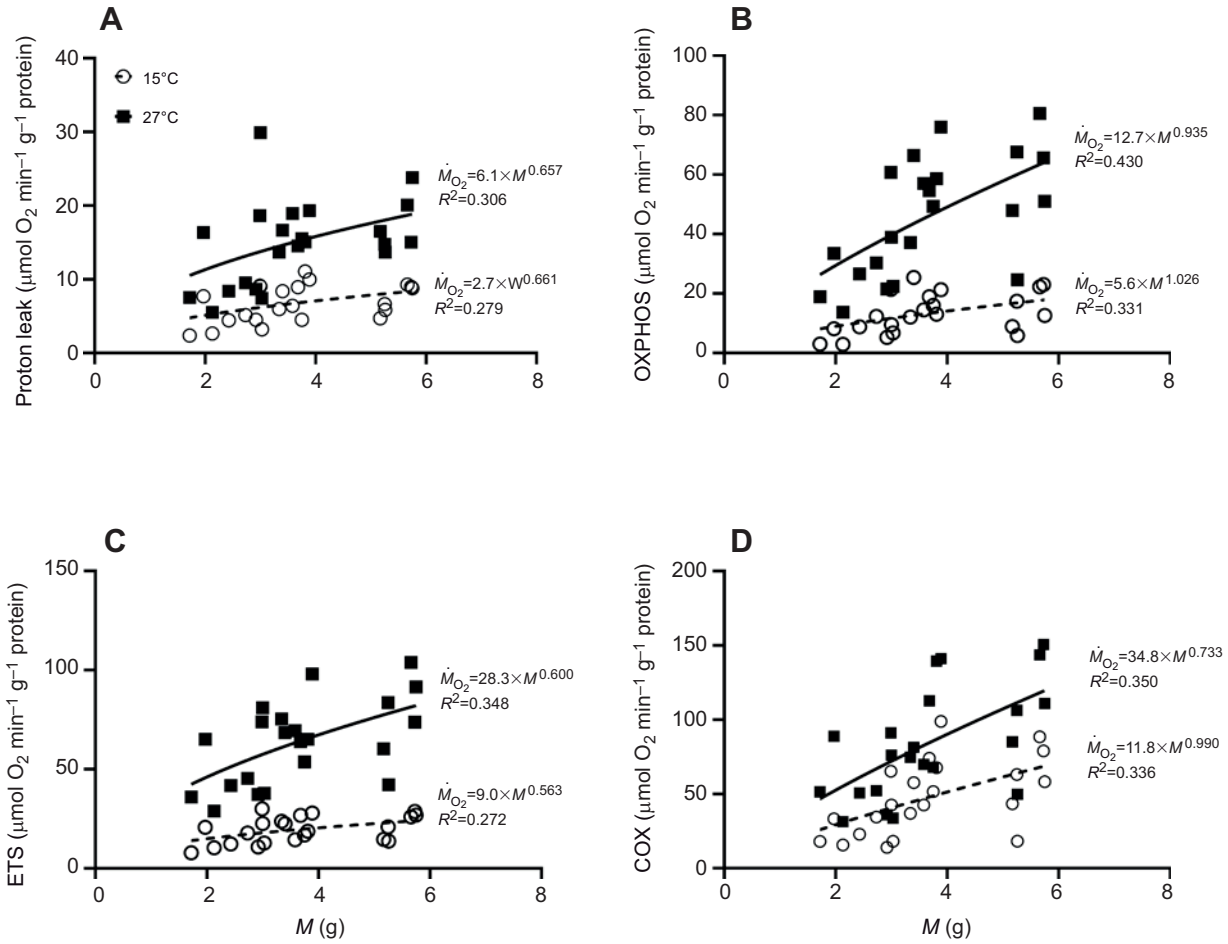

Fig. 2. Effect of body size (*M*, tissue wet mass) on mitochondrial respiration ($\dot{M}_{O_2}$) in mussels at normal and elevated temperature. (A) Proton leak respiration. (B) Active respiration indicative of OXPHOS. (C) Maximum activity of the complete mitochondrial ETS. (D) Maximum COX activity. Corresponding equations and R^2 values are given near the regression lines.

comparisons in mammals (Else and Hulbert, 1985b; Taylor, 1987; Porter and Brand, 1995b) and ectotherms (Savina et al., 1997). The observed allometry of cellular respiration was mostly explained by two factors: body size dependence of mitochondrial quantity and of mitochondrial proton leak. Thus, mitochondrial volume density and/or cristae surface area declined with increasing body mass in brain, liver, kidney, heart and skeletal muscle in mammals (Mathieu et al., 1981; Hoppeler et al., 1984; Else and Hulbert, 1985a,b; Taylor, 1987). The total mitochondrial membrane surface area of the major internal organs was proportional to body mass of mammals, with the metabolic scaling coefficient of 0.76 (0.59 when the skeletal muscle and the lung were excluded) (Else and Hulbert, 1985b). The maximal respiration of mitochondria in skeletal muscle of mammals was independent of the aerobic capacity of the particular muscle and of body mass at approximately 3–6 ml O_2 ml^{-1} mitochondria min^{-1} (Taylor, 1987).

In mammals (Porter, 2001) and birds (Brand et al., 2003), the rate of proton leak across mitochondrial membranes declines with increasing body mass. Studies on the relationship between mitochondrial activity and body mass in ectotherms are scarce, and correlation of their

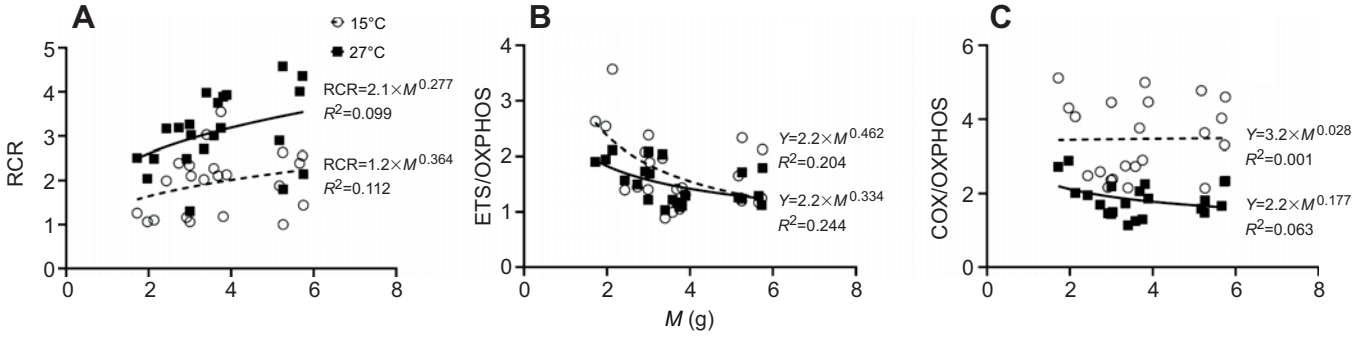

Fig. 3. Effect of body size (*M*, tissue wet mass) on RCR and reserve ETS and COX capacity in mussel mitochondria at normal and elevated temperatures. (A) RCR. (B) Reserve ETS capacity. (C) Reserve COX capacity. Corresponding equations and R^2 values are given near the regression lines.

cellular and mitochondrial respiration with body mass is less clear (see Sukhotin et al., 2017). There is no evidence of allometric scaling of ATP turnover or proton conductance across different species of ectotherms (Hulbert et al., 2002; Burpee et al., 2010). In three species of fish, the respiration rate of mitochondria from white muscles was independent of body mass, and the mitochondrial volume density in muscles declined with body mass in two of three studied fish species, indicating that mitochondrial traits in the muscle cannot explain the whole-body metabolic allometry in these species (Burpee et al., 2010). In three closely related species of frogs differing in adult body mass, OXPHOS respiration of mitochondria from liver tissue was also independent of animal size (Roussel et al., 2015). However, mitochondrial coupling efficiency markedly increased with body mass in frogs, leading to a higher ATP production in the larger frogs compared with the smaller species. This pattern was driven by a strong negative dependence of mitochondrial proton conductance on body mass (Roussel et al., 2015).

Our data obtained at the intraspecific level (in the bivalve *M. edulis*) unexpectedly demonstrated a pronounced allometric increase in both proton leak and OXPHOS activity of mitochondria from the digestive gland, one of the most metabolically active organs of the mussels. This finding was surprising because at the whole-organism level, the White Sea *M. edulis* show a typical pattern of a decrease in mass-specific metabolic rate with increasing body size when considered across all ages (b=−0.231 to −0.248) or within a single age group (e.g. for 5 year old mussels, b=−0.148 to −0.356) (Sukhotin and Pörtner, 2001; Sukhotin et al., 2002, 2006). A decline in the whole-animal mass-specific metabolic rate with increasing body mass has also been recorded in 3–4 year old mussels from the same population as studied in our present work (b=−0.333; A.S. and A.K., unpublished data). Exposure to elevated temperature stress did not affect the metabolic scaling of mitochondrial activities, so that even though the oxygen consumption rate increased with warming, the pattern of body mass-specific change in proton leak versus OXPHOS was preserved. These findings are at odds with previously published results of interspecific comparisons showing an allometric decline in proton leak and/or proton conductance with body mass and constant OXPHOS capacity of the mitochondria regardless of body size (Porter, 2001; Brand et al., 2003; Burpee et al., 2010; Salin et al., 2012a). It is worth noting that the body mass-dependent metabolic scaling of mussel mitochondrial activity found in the present study is disentangled from the effects of age as well as potential phylogenetic and lifestyle effects (present in cross-species comparisons) (Porter and Brand, 1993; Porter et al., 1996; Hulbert et al., 2002; Brand et al., 2003). In mussels, the metabolic scaling coefficient was different for OXPHOS and proton leak rates, with the allometric increase in OXPHOS almost twice as fast as the increase in proton leak. As a result, larger animals had ∼3 times higher OXPHOS rates relative to their smaller counterparts, while the proton leak rates increased only ∼2-fold over the same range of body mass. The differences in the metabolic scaling of OXPHOS and proton leak imply improved mitochondrial efficiency in the larger mussels, as the ATP synthesis capacity increases faster than the mitochondrial maintenance costs shown by the proton leak. The mitochondrial proton leak respiration reflects the baseline activity of the ETS needed to counteract all futile proton and cation cycles (not coupled to ATP production) and prevent depolarization of mitochondria (Rolfe and Brand, 1997). Mitochondrial proton leak contributes a large fraction (20–40%) of the basal maintenance costs in ectotherms including marine bivalves (Brand et al., 1991; Hulbert and Else, 1999; Hulbert et al., 2002; Cherkasov et al., 2006), so that lower proton leak rate translates into lower basal maintenance costs of the cell.

The differences in the metabolic scaling coefficients between OXPHOS and proton leak imply differences in the allometry of the underlying molecular processes that control mitochondrial activity in the actively phosphorylating versus resting states. In ectotherms, including molluscs and insects, OXPHOS is predominantly controlled by the activity of the ETS, with flux control coefficients of ∼0.7–1, indicating that 70–100% of the oxygen consumption of actively phosphorylating mitochondria is controlled by the ETS capacity (Chamberlin, 2004; Kurochkin et al., 2011; Ivanina et al., 2012, 2016). However, in mussels, the metabolic scaling coefficient is smaller for ETS activity than for OXPHOS (0.56–0.60 versus 0.93–1.02, respectively). This indicates that the allometry of OXPHOS cannot be fully explained by the ETS activity, and other processes, such as ATP synthase activity or adenylate transport (typically controlling 10–20% of the OXPHOS flux in molluscs; Kurochkin et al., 2011; Ivanina et al., 2012, 2016), might contribute. Interestingly, the differences in the metabolic scaling of OXPHOS and ETS result in a dramatic decrease in ETS reserve capacity with mussel body mass. Thus, in the small mussels, there is almost 2-fold ETS reserve capacity compared with the maximum OXPHOS flux, whereas in the larger mussels, the ETS reserve capacity is only ∼10%. This implies a greater metabolic flexibility of the OXPHOS flux in the smaller mussels that have potentially a larger span to upregulate OXPHOS (e.g. by increasing the activity of ATP synthase and/or adenylate transport) until the upper limit (set by the maximum ETS capacity) is reached.

The proton leak respiration of mitochondria of ectotherms including molluscs is controlled jointly by ETS activity (∼30–50% of control over the flux) and mitochondrial proton conductance (∼50–70% of control) (Chamberlin, 2004; Kurochkin et al., 2011; Ivanina et al., 2012, 2016). In endotherms, body mass-dependent changes in the mitochondrial proton conductance play an important role in metabolic scaling across different species (Porter and Brand, 1993; Mélanie et al., 2019). In the present study, we did not measure the proton conductance in mitochondria of the differently sized mussels. However, the similarity of the metabolic scaling coefficients of ETS and proton leak respiration suggests the allometry of the ETS as the most parsimonious explanation for the observed allometry of the proton leak in the mussel mitochondria. Further studies are needed to unequivocally resolve this and determine the mechanisms underlying the allometry of mitochondrial metabolism of mussels. However, regardless of the underlying mechanisms, our data show that the metabolic allometry at the mitochondrial level cannot explain the whole-organism metabolic scaling.

Mitochondrial energetics as an explanation for growth rate differences

Growth rate and duration (age) determine the body size attained by an organism. In our present study, all the mussels were of similar age but different sizes, reflecting different growth rates. Growth rate of mussels is influenced by numerous environmental factors, the most important being seasonal temperature variation and food availability (Bayne and Newell, 1983; Page and Hubbard, 1987; Handå et al., 2012). As all the experimental mussels in the current study were collected from the same aquaculture site, we assume they all were subjected to roughly the same environmental conditions. The results of the present study indicate that the difference in growth rate and, therefore, the ultimate size of mussels are associated with differences in the efficiency and ATP synthesis capacity of their mitochondria. Thus, mussels with faster growth (larger body size) showed an increase in OXPHOS and proton leak rates, ETS and COX activity

(indicating an overall improved mitochondrial performance) and higher RCR (indicating better mitochondrial coupling and potentially lower costs of mitochondrial maintenance at the same OXPHOS capacity) compared with slower growing specimens.

The link between mitochondrial functioning and growth performance has been reported in several endotherms and ectotherms species. Thus, the rate of mitochondrial energy production in muscle influenced the rate of body growth in pigs from the same genetic strain and in rats (Lutz, 2003). Lower rates of OXPHOS respiration and lower mitochondrial RCR were associated with reduced daily body mass gain in pigs growing from 18 to 28 kg. Furthermore, declines in proton leak-dependent respiration in pigs growing from 8 to 18 kg were associated with improvements in the efficiency of feed utilization (Lutz, 2003). The enhanced mitochondrial performance (and improved growth rates) was associated with higher levels of protein expression of the adenine nucleotide translocase type 1 (ANT1) in the muscles of pigs (Lutz, 2003), suggesting a role of the improved ATP and ADP transport in mitochondrial performance. Lower rates of mitochondrial proton leak and elevated RCR in muscle mitochondria were also associated with improved body mass gain to feed ratios in growing rats (Lutz, 2003). A study on poultry (broilers) and cattle (steers) showed that growth-efficient animals (with higher mass gain to feed intake ratios) had higher mitochondrial coupling (RCR) than growth-inefficient ones, while oxidative phosphorylation efficiency assessed by the ADP:O ratio did not differ between growth-efficient and -inefficient groups (Bottje and Carstens, 2009). These studies show that in endotherms, better coupling of electron transport to ATP production and less proton leakage are associated with higher growth efficiency.

In vertebrate ectotherms, similar to endotherms, mitochondrial capacity and/or efficiency are linked with growth performance. Thus, in the brown trout, *Salmo trutta*, food intake and growth performance are related to mitochondrial capacity (Salin et al., 2016). Low rates of feeding and growth were associated with high proton leak in liver and muscle mitochondria, and a lower coupling (RCR) of muscle mitochondria (Salin et al., 2016). In tadpoles of *Rana temporaria*, the maximal rate of ATP synthesis and phosphorylation efficiency (measured as ADP:O ratio) of the liver mitochondria were higher in the fast-growing individuals than in their slow-growing counterparts (Salin et al., 2012a,b). This was achieved through a higher ATP synthase activity in combination with a lower inner membrane proton leakage in fast-growing morphs, while the ETS capacity and the mitochondrial membrane potential did not differ between the groups. Unlike the results obtained for endotherms and fish (see above), RCR values were similar in slow- and fast-growing frogs (Salin et al., 2012a,b).

Enhanced mitochondrial performance in fast-growing mussels was observed in multiple bioenergetics-related traits including OXPHOS rate, ETS and COX activity of the mitochondria as well as proton leak. Therefore, despite improved mitochondrial coupling (reflected in higher RCR), the higher overall rate of the proton leak may contribute to higher cellular maintenance costs in faster-growing mussels. In ectotherms including molluscs, mitochondrial proton leak accounts for a large fraction (20–40%) of the cellular energy costs (Cherkasov et al., 2006). Therefore, unless compensated by lower mitochondrial volume density, an allometric increase in the mitochondrial proton leak rate might result in higher basal respiration rates of the larger (faster-growing) mussels. This is called the increased intake hypothesis (Burton et al., 2011), which states that a higher metabolic rate can support a higher sustained energy throughput, thus enabling greater assimilation of energy for growth and reproduction. Some studies (McCarthy, 2000) support this hypothesis, while others (Bayne, 1999, 2004) favour the alternative compensation hypothesis, which claims negative correlation of the standard metabolic rate with fitness traits, including growth rate. The rationale for the compensation hypothesis is that higher housekeeping metabolic requirements draw a larger proportion of energy from an organism's energy budget, thereby reducing the amount available for growth. These negative relationships with growth were shown for protein turnover rate, another major cellular ATP consumer, in bivalves including mussels (Bayne, 1999, 2004); however, determining whether this is also the case with the elevated proton leak requires further investigation.

It is worth noting that growth rate depends on both energy (food) consumption rate and growth efficiency, which reflects the ability and costs of energy conversion of consumed food into body mass (and therefore energy) gain. The latter may depend on physiological functions, such as feeding behaviour or digestion, and on cellular and biochemical processes, e.g. protein synthesis and turnover rate. Studies show that improved performance of mitochondria in the tissues of fast-growing animals can contribute to an increase in food consumption rate (Salin et al., 2016) as well as in growth efficiency (Lutz, 2003; Bottje and Carstens, 2009; Salin et al., 2012a,b). In our present study, we did not measure the food assimilation rate or growth efficiency of the mussels, and future studies are needed to determine the mechanisms by which the improved mitochondrial performance translates into the faster growth rates of mussels.

Our data demonstrate that mussels with faster growth (and thus larger body size) show an increase in OXPHOS rate, proton leak respiration rate, ETS and COX activity, and higher RCR compared with slower growing (smaller) individuals. The effect of body size per se and growth rate cannot be disentangled within the present data set. Earlier interspecific comparisons (that did not control for age or growth rates of animals) show that the allometric changes in tissue/cellular metabolic rate are mostly related to the mitochondrial quantity in tissues such as skeletal muscle (see review by Porter, 2001; Burpee et al., 2010). In intraspecific studies of allometry where the age of the experimental animals is constrained, the variation in body size is determined by the difference in the growth rates of the organisms. In such cases, the mitochondrial performance (i.e. intrinsic quality of the mitochondria) prevails in setting the allometric pattern in metabolism (e.g. Salin et al., 2012a; present study). Therefore, while the large-scale interspecific patterns of metabolic allometry might depend on mitochondrial density in cells, the inter-individual variation in growth performance, and consequently in body size, appears to be associated with mitochondrial capacity and cause different size-related patterns of metabolism. This hypothesis awaits further testing in a broader comparative framework using studies that control for age and/or growth rates of the individuals to fully elucidate the relationship between the mitochondrial and organismal metabolic allometry.

Acknowledgements
We thank Dr Vyacheslav Khalaman for providing us with mussels from the aquaculture site, Elena Likhareva who helped us with treatment of collected mussels, and two anonymous referees whose valuable and constructive comments have helped to improve the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.S., I.M.S.; Methodology: A.S., A.K., E.S., I.M.S.; Validation: A.K., E.S.; Formal analysis: A.K.; Investigation: A.S.; Resources: I.M.S.; Data

curation: A.K.; Writing - original draft: A.S., I.M.S.; Writing - review & editing: A.S., A.K., E.S., I.M.S.; Visualization: A.K.; Supervision: A.S., E.S., I.M.S.; Project administration: I.M.S.; Funding acquisition: A.S., I.M.S.

Funding

This study was carried out as part of the state research assignment of the Zoological Institute, Russian Academy of Sciences no. AAAA-A19-119022690122-5 and Russian Foundation for Basic Research (RFBR) grant no. 14-04-00466 to A.S. and A.K. The work was in part supported by the Deutsche Forschungsgemeinschaft project MitoBOX (award number 415984732, GZ: SO 1333/5-1) to I.M.S. and by the funding line Strategic Networks of the Leibniz Association within the scope of the Leibniz ScienceCampus Phosphorus Research Rostock (www.sciencecampus-rostock.de) to E.S. and I.M.S.

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