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Mitochondrial plasticity in brachiopod (*Liothyrella* spp.) smooth adductor muscle as a result of season and latitude

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Abstract Habitat temperature and mitochondrial volume density ($V_{V(mt,mf)}$) are negatively correlated in fishes, while seasonal acclimatization may increase $V_{V(mt,mf)}$ or the surface density of the mitochondrial cristae ($S_{V(im,mt)}$). The effect of temperature on invertebrate mitochondria is essentially unknown. A comparison of two articulate brachiopod species, *Liothyrella uva* collected from Rothera Station, Antarctica in summer 2007, and *Liothyrella neozelanica* collected from Fiordland, New Zealand in winter 2007 and summer 2008, revealed a higher $V_{V(mt,mf)}$ in the Antarctic brachiopod. The $S_{V(im,mt)}$ was, however, significantly lower, indicating the Antarctic brachiopods have more, less reactive mitochondria. *L. uva*, from the colder environment, had larger adductor muscles in both absolute and relative terms than the temperate *L. neozelanica*. Furthermore, a seasonal comparison (winter vs. summer) in *L. neozelanica* showed that the absolute and relative size of the adductor increased in winter, $V_{V(mt,mf)}$

was unchanged, and $S_{V(im,mt)}$ was significantly increased. Thus, seasonal acclimatization to the cold resulted in the same number of more reactive mitochondria. *L. neozelanica* was clearly able to adapt to seasonal changes using a different mechanism, i.e. primarily through regulation of cristae surface area as opposed to mitochondrial volume density. Furthermore, given the evolutionary age of these living fossils (i.e. approximately 550 million years), this suggests that mitochondrial plasticity has roots extending far back into evolutionary history.

Introduction

Temperature has significant effects on the physiology of ectotherms and is often seen as a master regulator affecting the whole animal on a number of levels including muscle function. Muscle is a highly plastic tissue and a host of compensatory mechanisms on a number of levels ranging from molecular through to organelles are employed to maintain function (Egginton and Sidell 1989; Johnston 1993; Sanger 1993; Watabe 2002). In fish, mitochondria are subject to reductions in efficiency as temperature decreases because increased unsaturated fatty acids are needed to maintain mitochondrial membrane fluidity at low temperatures (Logue et al. 2000; Guderley 2004b), which leads to increased proton leak across the membrane (Brand et al. 1991; Guderley 2004a). The thermodynamic effects of reduced temperature also lead to a reduction in enzyme activity (Sanger 1993; Guderley 2004b). Compensatory mechanisms include improved catalytic efficiency by the expression of different enzyme isoforms (Crockett and Sidell 1990; Guderley 2004b), increasing the mitochondrial volume density (Sanger 1993; Johnston et al. 1998) and in one study, the mitochondrial cristae surface density, i.e. the

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“amount” of cristae packed within a mitochondrion also increased in the cold (St-Pierre et al. 1998).

Although this is well characterised in fish, it is largely unknown whether the effects and mechanisms are the same in invertebrates. At best, only a handful of studies have examined the effects of temperature on mitochondrial plasticity in invertebrates. One laboratory study measured mitochondrial density in different populations of the same polychaete (*Arenicola marina*) from different latitudes, i.e. the North Sea and the White Sea, in response to thermal acclimation. A 2.4-fold increase was found in the mitochondrial volume density in the White Sea *A. marina* compared to the North Sea specimens (Sommer and Pörtner 2002). The cristae surface density was not measured in their study and is seldom measured in the majority of studies concerning temperature. Therefore, the possibility that polychaetes also increase the cristae surface density in their mitochondria in the cold cannot be excluded.

More recent work has investigated mitochondrial volume density in the Antarctic limpet *Nacella concinna* from different latitudes in the Southern Ocean (Morley et al. 2009). There was evidence of mitochondrial plasticity in the foot muscle. However, there was no evidence for the expected change in mitochondrial volume density and the key response reported was through a change in mitochondrial cristae surface area (Morley et al. 2009). While the results of this study are interesting, in particular, because they demonstrated that even stenothermal Antarctic invertebrates can exhibit a certain degree of mitochondrial plasticity, they are limited precisely because they come from a stenothermal invertebrate (although it can be argued that being intertidal, *N. concinna* is one of the more eurythermal Antarctic invertebrates).

To determine whether the paradigm of mitochondrial plasticity in response to temperature change is applicable to an even broader group of invertebrates, a completely different animal phylum was selected, the Brachiopoda. Furthermore, the present study provided the opportunity to investigate whether these invertebrates are capable of adaptation on both evolutionary and/or seasonal scales. This was possible because two species from the same genus, *Liothyrella uva* from the Antarctic Peninsula and *Liothyrella neozelanica* from Fiordland, New Zealand were studied. Articulate brachiopods such as *Liothyrella* spp. are filter feeders predominantly living off phytoplankton (Rhodes and Thayer 1991; Peck et al. 2005). They are traditionally described as common in polar regions, the deep sea and fiordic environments (James et al. 1992). In Fiordland, New Zealand, they occur at depths of 15–50 m. *L. neozelanica* experiences a broad range of temperatures in Fiordland, where winter temperatures may be as low as 9°C and summer maxima 18°C, although this varies with depth, and there is even greater variation close to the

surface (Cornelisen and Goodwin 2008). The Antarctic *L. uva* near Rothera, on the other hand, experiences very stable but extremely cold seawater temperatures of -1.8 to $+1.0$ °C.

Methods

Specimen collection

Antarctic *L. uva* (Fig. 1) were collected by SCUBA diving near Rothera Station, Adelaide Island ($67^{\circ}34.25'S$, $68^{\circ}08.00'W$) from 20 m depth in February 2007 ($N = 8$). Brachiopods were transported back to the UK where they were held for 3 months in a re-circulating seawater aquarium maintained at 0°C and a 16-h light/8-h dark photoperiod. Biological filtration and regular water changes maintained seawater quality. Brachiopods were fed an algal suspension (*Nannochloropsis* sp., Instant Algae[®]).

Temperate *L. neozelanica* (Fig. 1) were collected by SCUBA divers also from approximately 20–30 m depth in Doubtful Sound, New Zealand ($167^{\circ}02.91'E$, $45^{\circ}20.92'S$). Collections were made both in winter (July 2007, $N = 8$) when the seawater temperature was 11°C and in summer (February 2008, $N = 8$) when the water temperature was 17°C. The *L. neozelanica* were kept in a bucket of seawater for a maximum of 2 h before they were prepared for tissue fixation on shore at the University of Otago's Deep Cove field station in Doubtful Sound.

Brachiopod tissue preparation

Shell length was measured (± 0.01 mm) with vernier callipers, and wet weight (± 0.01 g) was determined both when the mantle was full of seawater and when empty. The anterior portions of both valves were cut away with scissors exposing the mantle cavity but leaving the majority of the two valves, the hinge and consequently the muscles intact and attached to both valves. This allowed in situ fixation of all muscles at their normal contracted length. Brachiopods were fixed for 24 h in 3% glutaraldehyde containing 0.05 M PIPES pH 7.6, 0.3 mM calcium chloride, 7 mM sucrose and 0.77 mM sodium azide. The osmolality was similar to that of seawater (800 mOsm kg^{-1}). After primary fixation, the brachiopods were washed three times with, and stored for 5 weeks in the same solution as earlier, without the glutaraldehyde and sodium azide.

Smooth adductor muscles were dissected free from their attachment points and care was taken not to destroy the natural form. Muscles were dried with tissue paper and weighed (± 2 mg) and then weighed again in fixative to determine the tissue density (1.018). Care was again taken

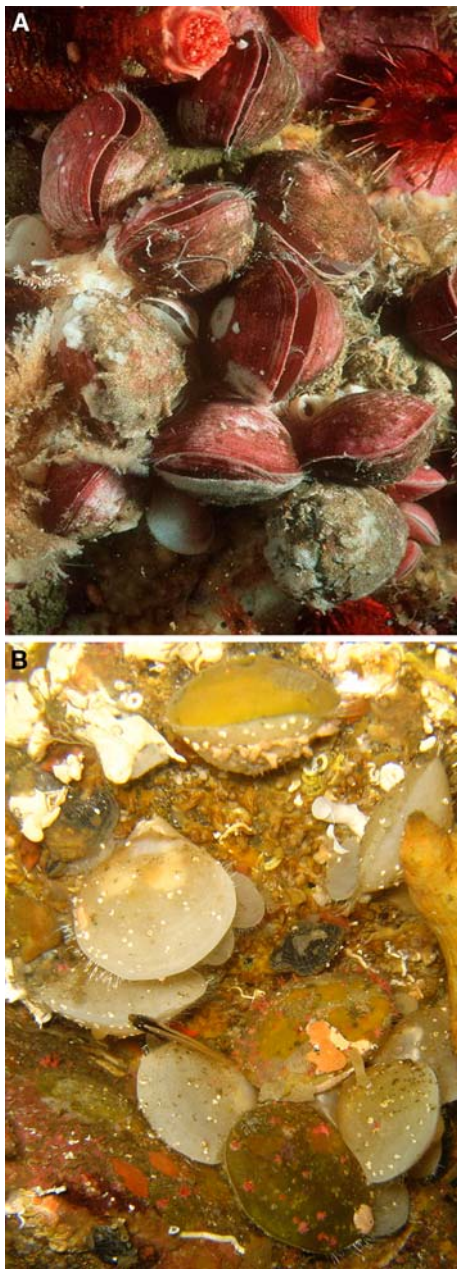


Fig. 1 **a** Antarctic brachiopod *Liothyrella uva*. **b** New Zealand brachiopod *Liothyrella neozelanica*

to ensure the tissue did not dry out significantly. Drying and weighing took approximately 15–20 s. Muscles were secondary fixed in 1% osmium tetroxide for 2 h, washed 3 times in the same storage buffer used earlier and left overnight. They were bulk stained in 0.05 M maleate buffered (pH 5.2) uranyl acetate for 2 h. Muscles were then serially dehydrated in 70, 90 and 100% ethanol solutions before being embedded in Quetol 651 and cured at 60°C for 48 h (Ellis 2002). Blocks were selected at random for semi-thin (0.5 μm) sectioning. One block containing a

whole adductor muscle was sectioned transversely at approximately half the length of the adductor muscle using a Reichert ultramicrotome. Sections were stained with methylene blue. Ultrathin sections (80 nm) were placed on copper grids (200 mesh). Grids were then stained for 5 min in uranyl acetate saturated 50% methanol and then for 5 min in lead citrate.

Morphometry

Methylene blue stained semi-thin sections were analysed using a Zeiss Axioskop at 100 \times magnification fitted with an Olympus DP70 digital camera that was connected to a computer. Frames were selected randomly by starting in the top left corner and sampling every 5th frame with ten frames sampled, i.e. 250 myocytes. The maximum cell diameter (μm) was determined to be the distance between the two most distant myocyte edges perpendicular to the longitudinal axis of the cell and was measured using the arbitrary distance function in the Olympus DP software, version 3.2.

The relative mitochondrial volume density ($V_{V(\text{mt},\text{mf})}$), given as a proportion of muscle fibre volume, was determined as per Morley et al. (2009). Briefly, point counting (Weibel 1979) at 4,400 \times magnification on a Zeiss EM902 transmission electron microscope, with a 2.12- μm -spaced 16-point grid was used. For each individual, 120 fields of view were analysed (Supplemental Fig. 2). Electron micrographs of individual mitochondria (8–15 per specimen, Fig. 2 supplementary material) at a magnification of 32,000 \times were taken for the estimation of surface density of the inner mitochondrial membrane ($S_{V(\text{im},\text{mt})}$), which was determined using line-intercept measurements (Weibel 1979). Absolute mitochondrial volume (per adductor muscle) was calculated by multiplying the adductor muscle weight by the proportion of mitochondria. The absolute inner mitochondrial membrane surface area (per adductor muscle) was calculated by multiplying the absolute mitochondrial volume by the relative surface area.

Statistics

Statistical analysis was performed using SigmaStat 3.5. All data were checked for normality using a Kolmogorov–Smirnov test (with Lilliefors’s correction) and equal variances. For the data that were normal and had equal variances, one-way ANOVAs were used to test for significant species effects, while post hoc Holm–Sidak tests were used to determine significant differences between groups. Normal data with unequal variances (i.e. absolute volume density and absolute cristae surface area) were tested for significance (without transformation) using a Kruskal–Wallis one-way ANOVA on ranks and Tukey

tests for differences between groups. All values are given as the mean \pm SE of the mean.

Results

Significant differences existed between the sizes (measured as shell length) of the *Liothyrella* spp. sampled ($P < 0.05$, $F = 18.5$, $df = 2$). The Antarctic *L. uva* were smaller than *L. neozelanica* sampled in winter, and both of these were smaller than the *L. neozelanica* collected in the summer (Table 1). In contrast, adductor wet weight was significantly greater in *L. uva* than in summer *L. neozelanica* ($P < 0.05$), while winter *L. neozelanica* were intermediate in size and not significantly different from the other species/group (Table 1). As a consequence, there were significant differences among all groups in the adductor muscle wet weight relative to the size ($P < 0.05$, $F = 12.3$, $df = 2$) of the brachiopod (Table 1), which decreased from *L. uva* to the winter *L. neozelanica* to the smallest ratio in summer *L. neozelanica*. The Antarctic *L. uva* myocyte diameter was 27% greater than in the winter *L. neozelanica* ($P < 0.05$) and 18% larger than in the summer *L. neozelanica* ($P < 0.05$). There were no significant differences between the diameters of myocytes from winter or summer *L. neozelanica*, indicating no cellular hypertrophy as a result of seasonal acclimatization (Table 1).

The muscle fibre mitochondrial volume density ($P < 0.05$, $F = 4.2$, $df = 2$), was significantly higher in *L. uva* than in summer *L. neozelanica* (Table 2). The winter *L. neozelanica* $V_{V(mt,mf)}$ was intermediate between the two and not significantly different from either group, but much closer to the summer *L. neozelanica* value. Absolute

mitochondrial volume was also significantly different among the groups ($P < 0.05$, $H = 15.3$, $df = 2$), highest in *L. uva* and lowest in summer *L. neozelanica*. The winter *L. neozelanica* was again intermediate (Table 2). This was due mainly to *L. uva* having larger adductor muscles than *L. neozelanica*, indicating increased myocyte size in the cold (Table 1).

The cristae surface density differed significantly between groups ($P < 0.05$, $F = 8.97$, $df = 2$). Although $S_{V(im,mt)}$ was approximately the same in *L. uva* and summer *L. neozelanica*, the winter *L. neozelanica* had significantly greater relative cristae density than either of these (Table 2). The absolute cristae surface area was also significantly different between groups ($P < 0.05$, $H = 11.7$, $df = 2$), with it being significantly higher in *L. uva*, than summer *L. neozelanica* again due principally to the greater adductor muscle wet weight (Table 1), and intermediary in winter *L. neozelanica* (Table 2). The surface area of mitochondrial cristae per unit muscle fibre volume can be used as an estimate of relative aerobic capacity (see Johnston et al. 1998). This measure found no significant differences among the groups ($P = 0.07$, $F = 3.0$, $df = 2$), i.e. it was similar in *L. uva* and winter *L. neozelanica*, but it tended to be lower ($P = 0.06$) in summer *L. neozelanica* (Table 2).

Discussion

The sizes of the brachiopods differed significantly among the groups studied here. In the absence of any data on the relationship between muscle size and shell size, differences were corrected using the adductor wet weight to shell

Table 1 Brachiopod gross morphological data

	Shell length (mm)	Adductor wet weight (mg)	Weight-to-length ratio	Myocyte diameter (μm)
<i>L. uva</i>	35.6 \pm 1.38 ^a	69.4 \pm 7.40 ^a	1.92 \pm 0.16 ^a	3.57 \pm 0.16 ^a
<i>L. neozelanica</i> (winter)	40.8 \pm 1.04 ^b	55.1 \pm 6.78 ^{a,b}	1.35 \pm 0.16 ^b	2.81 \pm 0.14 ^b
<i>L. neozelanica</i> (summer)	48.3 \pm 1.91 ^c	40.7 \pm 5.89 ^b	0.85 \pm 0.14 ^c	3.02 \pm 0.12 ^b

Dissimilar letters indicate significant differences ($P < 0.05$). Mean \pm SE. $N = 8$ for each group

Table 2 Mitochondrial parameters from brachiopod adductor muscle in relative and absolute terms

	$V_{V(mt,f)}$	Mitochondrial volume (μm^3)	$S_{V(im,mt)}$ ($\mu\text{m}^2 \mu\text{m}^{-3}$)	Cristae surface area (μm^2)	Aerobic capacity ($\mu\text{m}^2 \mu\text{m}^{-3}$)
<i>L. uva</i>	0.013 \pm 0.0010 ^a	0.84 \pm 0.043 ^a	25.9 \pm 1.32 ^a	21.5 \pm 1.43 ^a	0.34 \pm 0.028
<i>L. neozelanica</i> (winter)	0.009 \pm 0.0007 ^{a,b}	0.51 \pm 0.082 ^{a,b}	35.1 \pm 1.99 ^b	18.0 \pm 3.11 ^a	0.33 \pm 0.030
<i>L. neozelanica</i> (summer)	0.009 \pm 0.0016 ^b	0.26 \pm 0.025 ^b	26.7 \pm 1.74 ^a	6.96 \pm 0.78 ^b	0.23 \pm 0.042

Dissimilar letters indicate significant differences ($P < 0.05$). Mean \pm SE. $N = 8$ for each group

length ratio. Using this measure, the Antarctic *L. uva* had 21% larger adductor muscles than the winter *L. neozelanica*, although the former were 13% smaller. This could be the result of cold-induced muscular hypertrophy or mechanical and/or other species differences. There was, however, an even greater seasonal change in adductor muscle size in *L. neozelanica*, with summer specimens having 26% smaller adductor muscles than winter samples although they were 16% larger. The seasonal increase in adductor wet weight observed here in *L. neozelanica* was due primarily to an increase in the number of myocytes (cellular hyperplasia), as opposed to increased myocyte size (cellular hypertrophy). Although seasonal hypertrophy of muscle fibres in ectotherms has been investigated only rarely, for example in halibut (Haugen et al. 2006), cold-induced hypertrophy is not an entirely new phenomenon, having been previously documented in fish and mammals (Sidell 1980; Sidell and Moerland 1989). Cold-induced hypertrophy compensates for a reduction in the contraction rate at low temperatures by increasing the muscle fibre number (Rome 1990).

The relative mitochondrial volume density in both *L. uva* and *L. neozelanica* was low (0.9–1.3%) compared to more active animals such as squid mantle muscle (50%, Moon and Hulbert 1975), and fish red skeletal muscle (8–50%, Johnston et al. 1998). It was in a similar range to the volume density of mitochondria previously recorded in slower moving or sessile invertebrates, e.g. 1.8–4.5% in polychaete body wall musculature (*A. marina*, Sommer and Pörtner 2002), 0.9–1.3% in scallops (*Aequipecten opercularis*, Philipp et al. 2008), and 1.3–2.2% in limpets (*N. concinna*) and clams (*Laternula elliptica*, Morley et al. 2009). Analogous to previous findings in polychaete worms, the evidence suggests that con-generic brachiopods increase their mitochondrial volume density in response to the extreme cold of the Antarctic. Whether this trend is consistent across the phylum, Brachiopoda remains to be examined. Nevertheless, it seems reasonable to conclude that temperature is the principal factor affecting mitochondrial density as these two species are from the same genus, thus eliminating phylogenetic differences. They also share essentially the same habitat and lifestyle (Peck 2001).

The Antarctic *L. uva* shows only very small seasonal changes in basal metabolic rate (Peck et al. 1987). However, feeding raises metabolic rate in this species 1.6-fold (Peck 1996), and this means summer *L. uva* might be expected to increase mitochondrial densities to meet the extra activity costs associated with increased feeding. Several pieces of evidence argue against this. First and foremost, any changes in mitochondrial volume density associated with feeding would be expected to take place in the gut and digestive gland, not in muscle. Secondly, although the quantity of food in winter is drastically

reduced, so that it does not meet basal metabolic requirements (Peck et al. 1987), the unchanged basal metabolic rate indicates that *L. uva* decouples growth from feeding, increasing in size in winter on stored protein/glycoprotein reserves from summer (Peck and Holmes 1989; James et al. 1992), i.e. total individual biomass is converted into increased size (Peck et al. 1997). Thirdly, applying the same principle that metabolic rate is up-regulated in the summer in response to enhanced food supply would mean that *L. neozelanica*, collected in the summer should have higher mitochondrial densities as that is the primary time of feeding for this species too, as reflected by the higher chlorophyll concentrations in summer (Goebel et al. 2005). There were, however, no differences in the mitochondrial densities of winter and summer *L. neozelanica*. Thus, it is most likely that temperature is the primary determinant of mitochondrial volume density only when comparing different *Liothyrella* species.

There was evidence for two different strategies for preserving similar myocyte aerobic capacities in the cold in the current study. Evolutionary adaptation of mitochondria to changes in habitat temperature was marked by increased mitochondrial volume while the amount of cristae packed within the mitochondria was maintained (*L. uva* vs. *L. neozelanica*). Seasonal changes in habitat temperature were countered by increased cristae packing within the mitochondria while the mitochondrial volume stayed the same. These results seasonal changes are similar to recent findings in the Antarctic limpet *N. concinna* (Morley et al. 2009). In that study limpets acclimated to 0°C showed only minor changes in mitochondrial volume density compared to 3°C acclimated limpets, but significant changes in mitochondrial cristae surface density were observed (Morley et al. 2009). The temperature difference in the current study was twice as large, but still modest. A larger temperature difference may reveal even greater differences in cristae surface density, or indeed differences in the volume density.

The different forms of plasticity, i.e. volume density and cristae surface density, may have different costs associated with them in different thermal regimes. Changes in cristae surface density due to seasonal acclimatization in *L. neozelanica* may be a more efficient way of up-regulating the reactive surface for oxidative phosphorylation in individual mitochondria, eliminating some of the costs associated with making mitochondria de novo. Furthermore, this strategy also prevents a loss in volume of the contractile apparatus if the size of the myocytes is to stay the same, as it appears to in *L. neozelanica*, analogous to the optimal fibre size hypothesis (Johnston et al. 2003). The costs associated with this strategy may be an increase in basal energy requirements and an increase in the oxidative stress associated with these more reactive mitochondria

(Guderley 2004a). However, brachiopods apparently employ low energy, high efficiency energy utilisation strategies (James et al. 1992). Changing cristae density may ultimately be less costly than mitochondrial biogenesis.

Simply increasing the number (i.e. volume density) of mitochondria but not their reactive surface may mitigate oxidative stress problems, and this may be important in a brachiopod (*L. uva*) that inhabits an area of high ambient oxygen levels and recognised high oxidative stress (Abele and Puntarulo 2004). Another hypothesis states that mitochondrial proliferation in the cold may be the result of diffusional limitations (Sidell and Hazel 1987; Guderley 2004a; Kinsey et al. 2007). As temperature decreases, so too does the rate of diffusion of substrates and metabolites within the intracellular milieu. Proliferation in the cold means that more less-efficient mitochondria compensate for this limitation as the mitochondria are closer to the point of ATP demand. This would be particularly advantageous for the Antarctic *L. uva* where diffusion of ATP will not only be slower, but *L. uva* also has myocytes 20% greater in diameter and 40–60% greater in volume.

This study is the first to find evidence of mitochondrial plasticity in articulate brachiopods, a phylum with representatives that appear to have existed on Earth with unchanged morphology for over 550 million years, stretching back to the early Cambrian (James et al. 1992). The Brachiopoda, Mollusca, Annelida and Vertebrata, therefore, have all been shown to exhibit some degree of mitochondrial plasticity and three of the four phyla have ancestors reaching back to the Cambrian/Pre-Cambrian. This obviously has evolutionary implications and indicates that mitochondrial flexibility probably has deep evolutionary roots. Just how the volume density and cristae surface density are regulated at a molecular level in response to temperature is still largely unknown. The recent finding that peroxisome proliferator activated receptor gamma co-activator 1-alpha (PGC-1 α) is a master regulator of mitochondrial biogenesis in mammalian and fish muscle (Houten and Auwerx 2004; Urschel and O'Brien 2008) makes it a prime candidate for examining the effects of temperature on mitochondrial proliferation in brachiopods. Irrespective of the regulation mechanisms, this study has demonstrated different seasonal and evolutionary mechanisms of compensation of mitochondrial function to low temperature in brachiopod adductor muscle.

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