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Title: Mitochondrial plasticity in brachiopod (Liothyrella sp.) smooth adductor muscle as a result of season and latitude

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Abstract: In fishes a negative correlation exists between habitat temperature and the mitochondrial volume density (Vv(mt,mf)), while seasonal acclimatization may increase Vv(mt,mf) or the surface density of the mitochondrial cristae (Sv(im,mt)). The affect of temperature on invertebrate mitochondria is essentially unknown. A comparison of two articulate brachiopod species, Liothyrella uva collected from Rothera Station, Antarctic in summer 2007, and Liothyrella neozelanica from Fiordland New Zealand collected in winter 2007 and summer 2008, revealed a higher Vv(mt,mf) in the Antarctic brachiopod. The Sv(im,mt) was however, significantly lower, indicating the Antarctic brachiopods have more less reactive, mitochondria. Liothyrella 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, Vv(mt,mf) was unchanged, however Sv(im,mt) was significantly increased. Thus, seasonal acclimatization to the cold resulted in the same number of more reactive mitochondria. Liothyrella 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 that extend far back into evolutionary history.

Dear Sir

Firstly, thank you very much for your positive reply regarding our manuscript "Mitochondrial plasticity in brachiopod (*Liothyrella* sp.) smooth adductor muscle as a result of season and latitude" **(MABI-D-09-00514)**. We have carefully read through your and the reviewer's comments on our manuscript and considered them accordingly. Below is our response to some of the points made.

To date, mitochondrial cold proliferation has only been described in one group of ectotherms, fish. As such, this is the logical departure point for this study. Our study is the first to look at mitochondrial characteristics as a function of temperature in invertebrates in both a long-term (adaptational) and short-term (seasonal) sense. Therefore we are forced to compare our data with those from fish as there are no other data to compare with. Nevertheless, we do agree that fishes and brachiopods are vastly different animals, and any comparison has its' limitations. Consequently we have reined in our discussion and eliminated many of the comparisons with fish, except where we think appropriate.

The section regarding the two different forms of mitochondrial plasticity has also been condensed and is hopefully less convoluted as a result.

Spring, i.e. September to November, is the time of the largest planktonic bloom in Doubtful Sound as indicated by the greatest chlorophyll concentrations (Goebel, 2005), however there is a second, smaller bloom that usually occurs between January and March, which was the time we collected our brachiopods.

Figures 2A and B are now supplemental figures and the data from figures 3-6 are presented in two tables.

All other comments have been duly noted and the manuscript changed accordingly.

Reviewer 2 raised a valid concern with respect to scaling of organs in brachiopods. Evidence from mammals indicates that the relationship between muscle/organ mass and size is isometric (Hoppeler & Weibel 2000, Acta Phys Scan 168, 445-56, Mathieu et al 1981, Resp Physiol 41, 113-125). Obviously, extending this directly to brachiopods would be tenuous at best, and we concede that the relationship between the length of the brachiopods and the adductor muscle wet weight may not necessarily be isometric. But in the absence of any data of our own, or in the literature on organ scaling in invertebrates, we feel that our attempt to correct for size effects is valid. Deriving scaling exponents from our data are precluded, primarily by the very narrow size range we used in order to avoid this problem, i.e. 37-54mm for *L. neozelanica*. Furthermore, we consider the differences seen here to be of sufficient magnitude (i.e. a 27% larger absolute muscle mass in brachiopods that are 16% smaller) to sufficiently qualify as evidence of cold-induced muscular hypertrophy. Nevertheless, we have reined in our speculative discussion in accordance with reviewer 2's wishes.

We do not have data for other tissues as this was not the focus of the study. As a consequence, we are not able to give a condition index. We do not think this would have any major impact on our findings. As pointed out in the discussion, if

condition/nutritional state were to have an effect, it would be much more likely to be seen in the digestive organs than the adductor muscle.

Reviewer 2's specific comments:
1) The referee is correct in that these brachiopods do lay down stored reserves in the summer and convert these to cell division and increase in structural materials in winter. However, unlike many other groups (that do store lipids and carbohydrates), these stores are proteins or glycoproteins (James et al 1992), and thus, although they are almost certainly transformed form storage to structural, they are not transformed from lipids and CHO to protein. *Liothyrella uva* increases in shell length in the winter more than the summer (about 4 times as much growth in winter than summer, (Peck et al 1997, Phil Trans R. Soc 352, 851-858), and this cycle in increase in linear dimension occurs completely out of phase with changes in mass.

Hopefully we have made this clearer with the following alteration in the third paragraph of the discussion: Secondly, although the quantity of food in winter is drastically reduced, such that it is insufficient to met the basal metabolic requirements (Peck et al. 1987), the unchanged basal metabolic rate indicates that *L. uva* decouple growth from feeding, growing in size in winter on stored protein/glycoprotein reserves from summer (Peck et al. 1989, James et al. 1992), i.e. total animal mass is converted into increased size (Peck et al. 1997).

2) Although evidence in mammals indicates that there is a direct linear relationship between mitochondrial volume density and oxygen consumption (Mathieu et al 1981, Resp Physiol 41, 113-125), we are aware that there may not necessarily be a linear relationship between volume density, cristae surface density and the myocyte aerobic capacity in brachiopods. Our simple assertion is that if either volume density or cristae surface density increase then an increase in the myocyte aerobic capacity will follow.

3) Giving the mitochondrial volume (μm^3) is essential as this is a parameter that could affect not only the aerobic capacity of the muscle, but ultimately the respiration rate of the whole animal. This parameter demonstrates that although the winter brachiopods were smaller, they had larger adductor muscles. A larger muscle mass means they had more mitochondria, which would have an affect on the metabolic rate. This is a fact that has no dependence on whether the increase in adductor weight scales isometrically, or was the result of cold induced hypertrophy.

4) Through shortening and condensing the discussion we were able to reduce the number of references from 55 to 38.

5) The discussion has also been shortened by halving the section on hypertrophy to one paragraph. Nevertheless we still think it is important to point out the significance of the differences in shell length and adductor muscle wet weight between the animals, and that if not conclusive evidence, then it is very suggestive of cold-induced hypertrophy.

We hope you consider our remarks favourably. I look forward to another speedy and positive reply, and the possibility of publishing in your illustrious journal.

Yours sincerely
Glenn Lurman

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

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Keywords: brachiopod, Antarctic, myocyte diameter, mitochondria, cristae, Liothyrella, temperature

Abstract

In fishes a negative correlation exists between habitat temperature and the mitochondrial volume density ($Vv_{(mt,mf)}$), while seasonal acclimatization may increase $Vv_{(mt,mf)}$ or the surface density of the mitochondrial cristae ($Sv_{(im,mt)}$). The affect of temperature on invertebrate mitochondria is essentially unknown. A comparison of two articulate brachiopod species, *Liothyrella uva* collected from Rothera Station, Antarctic in summer 2007, and *Liothyrella neozelanica* from Fiordland New Zealand collected in winter 2007 and summer 2008, revealed a higher $Vv_{(mt,mf)}$ in the Antarctic brachiopod. The $Sv_{(im,mt)}$ was however, significantly lower, indicating the Antarctic brachiopods have more less reactive, mitochondria. *Liothyrella 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, $Vv_{(mt,mf)}$ was unchanged, however $Sv_{(im,mt)}$ was significantly increased. Thus, seasonal acclimatization to the cold resulted in the same number of more reactive mitochondria. *Liothyrella 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 that extend 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; Sänger 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 (Sänger 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 (Sänger 1993; Johnston et al. 1998) and in one study, the mitochondrial cristae surface density, i.e. the "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 exist that have looked at the effects of temperature on mitochondrial plasticity in invertebrates. One laboratory based study looked at mitochondrial density in different populations of the same species of marine mud worm (*Arenicola marina*) from different latitudes, i.e. the North Sea and the White Sea in response to thermal acclimation. An increase of 2.4-fold was found in the mitochondrial volume density in the White Sea *A. marina* compared to the North Sea *A. marina* (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 annelids 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 within 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 invertebrates (although it can be argued that being intertidal, *N. concinna* are one of the more eurythermal Antarctic invertebrates).

To see 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* are filter feeders predominantly living off phytoplankton (Rhodes & 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. *Liothyrella neozelanica* experience a broad range of temperatures in Fiordland, where winter temperatures may drop to 9°C and summer maxima climb to 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, experience very stable but extremely cold seawater temperatures of -1.8 - +1.0°C.

Methods

Specimen Collection

Antarctic *Liothyrella uva* (Fig. 1) were collected by SCUBA diving near Rothera Station, Adelaide Island (67°34.25'S, 68°08.00'W) from 20 m depth in February 2007 (N = 8). Brachiopods were then transported back to the UK where they were held for 3 months in a re-circulating seawater aquarium maintained at 0°C and a 16:8 light:dark photoperiod. Biological filtration and regular water changes maintained seawater quality whilst the addition of an algal solution (*Nannochloropsis*, Instant Algae ®) supplemented the natural food in the seawater.

Temperate *Liothyrella neozelanica* (Fig. 1) were collected by SCUBA divers also from approximately 20-30 m depth in Doubtful Sound, New Zealand (167°02.91'E, 45°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. Collected *Liothyrella neozelanica* were kept in a bucket of seawater for a maximum of 2 hours before they could be prepared for tissue fixation on shore at the University of Otago 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) were 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 hours 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 above, 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 to ensure the tissue did not dry out significantly. Drying and weighing took approximately 15-20 sec. Muscles were secondary fixed in 1% osmium tetroxide for 2 hrs, washed 3 times in the same storage buffer used above and left overnight. They were bulk stained in 0.05 M maleate buffered (pH 5.2) uranyl acetate for 2 hrs. Muscles were then serially dehydrated in 70, 90 and 100% ethanol solutions before being embedded in Quetol 651 and cured at 60°C for 48hrs (Ellis 2002). Blocks were selected at random for semithin (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 100x 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 ($Vv_{(mt,mf)}$), given as a proportion of muscle fibre volume, was determined was determined as per Morley et al. (2009). Briefly, point counting (Weibel 1979) at 4400x magnification on a Zeiss EM902 transmission electron microscope, with a 2.12 µm spaced 16 point grid was used. For each animal 120 fields of view were analysed (Supplemental Fig. 2). Electron micrographs of individual mitochondria (8 to 15 per animal, Fig 2 supplementary material) at a magnification of 32000x were taken

for the estimation of the surface density of the inner mitochondrial membrane ($Sv_{(im, mt)}$), 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 Kolmogorov-Smirnov test (with Lilliefor's correction) and equal variances. For the data were normal and had equal variances, a one-way ANOVAs were used to test for significant species effects, while *post-hoc* Holm-Sidak tests were used to determine significant 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 to test for differences between groups. All values are given as the mean ± standard error of the mean (SE).

Results

Significant differences existed between the sizes (measured as shell length) of the *Liothyrella* sampled ($p < 0.05$, $F = 18.5$, d.f. = 2). The Antarctic *L. uva* were smaller than *L. neozelanica* sampled in winter, and both of these were smaller than *L. neozelanica* sample collected in the summer (Table 1). Opposite to this, adductor wet weight was significantly greater in *L. uva* than summer *L. neozelanica* ($p< 0.05$), while winter *L. neozelanica* was intermediary and not significantly different to either (Table 1). As a consequence, there were significant difference between all groups in the adductor muscle wet weight relative to the size ($p < 0.05$, $F = 12.3$, d.f. = 2) of the brachiopod (Table 1), which declined from *L. uva* through winter *L. neozelanica* to the smallest ratio in summer *L. neozelanica*. The Antarctic *L. uva* myocyte diameter was 27% greater than winter *L. neozelanica* ($p < 0.05$) and 18% larger than summer *L. neozelanica* ($p < 0.05$). No significant differences were seen between the diameter of myocytes from winter or summer *L. neozelanica*, indicating no cellular hypertrophy as a result of seasonal acclimatization (Table 1).

A significant difference was seen in the muscle fibre mitochondrial volume density ($p < 0.05$, $F = 4.2$, d.f. = 2), which was significantly higher in *L. uva*, than summer *L. neozelanica* (Table 2). The winter *L. neozelanica* $Vv_{(mt,mf)}$ 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 between the groups ($p < 0.05$, $H = 15.3$, d.f. = 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 ($p < 0.05$, $F = 8.97$, d.f. = 2). Although $Sv_{(im,mt)}$ 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 ($p < 0.05$, $H = 11.7$, d.f. = 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 between the groups ($p = 0.07$, $F = 3.0$, d.f. = 2), i.e. it was similar to *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 between the groups studied here. In the absence of any data on the relationship between muscle size and shell size, differences were corrected for using the adductor wet weight to shell length ratio. On this measure the Antarctic *L. uva* had 21% larger adductor muscles than the winter *L. neozelanica*, although they 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 mantel 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 (*Arenicola marina*, Sommer and Pörtner, 2002), 0.9-1.3% in scallops (*Aequipecten opecularis*, Philipp et al. 2008), and 1.3-2.2% in limpets (*Nacella concinna*) and mud 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 the mitochondrial density as these two species are from the same genus, which eliminates many differences in phylogeny. They share also 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 by 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, such that it is insufficient to met the basal metabolic requirements (Peck et al. 1987), the unchanged basal metabolic rate indicates that *L. uva* decouple growth from feeding, growing in size in winter on stored protein/glycoprotein reserves from summer (Peck and Holmes 1989; James et al. 1992), i.e. total animal mass is converted into increased animal 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 densities as that is the primary time of feeding for this species too, as reflected by the higher chlorophyll concentrations seen in summer (Goebel et al. 2005).

There were, however, no differences seen in the density 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.

Evidence of two different strategies for preserving similar myocyte aerobic capacities in the cold was evident 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. The results concerning seasonal changes are similar to recent findings in the Antarctic limpet *Nacella 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. However, significant changes in the mitochondrial cristae surface density were observed in response to an acclimation difference of only 3°C (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 the 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 the different thermal habitats. 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 indeed 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 the basal energy requirements and an increase in the oxidative stress associated with these more reactive mitochondria (Guderley 2004a). However, brachiopods have been described as employing low energy, high efficiency energy utilisation strategies (James et al. 1992). Changing cristae density may ultimately be a less costly mechanism 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 of importance in an animal (*L. uva*) that inhabits an area of high ambient oxygen levels and recognised high oxidative stress in some species (Abele and Puntarulo 2004). Another hypothesis put forward

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 have 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, and this is a phylum that has 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 Brachopoda, 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, stretching back to the Pre-Cambrian. 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 PGC-1α is a master regulator of mitochondrial biogenesis in mammalian and fish muscle (Urschel and O'Brien 2008, Houten and Auwerx 2004) 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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Figures

Fig 1. **A)** Antarctic brachiopod *Liothyrella uva*. **B)** New Zealand brachiopod *Liothyrella neozelanica*.

Supplemental Fig. 2. **A)** Electron micrograph of adductor muscle from *Liothyrella uva*. Mitochondria are indicated with arrowheads. The scale bar represents 5µm. **B)** Electron micrograph of a single mitochondrion in *Liothyrella uva* adductor muscle. Scale bar represents 1µm.

Tables

Table 1. Brachiopod gross morphological data. Dissimilar letters indicate significant differences ($p < 0.05$). Mean ± SE. $N = 8$ for each group.

	Shell Length (mm)	Adductor Wet Weight (mg)	Weight to Length Ratio	Myocyte Diameter (μm)
L. uva	35.6 ± 1.38 [a]	69.4 ± 7.40 [a]	1.92 ± 0.16 [a]	3.57 ± 0.16 [a]
L. neozelanica (Winter)	40.8 ± 1.04 [b]	55.1 ± 6.78 [a,b]	1.35 ± 0.16 [b]	2.81 ± 0.14 [b]
L. neozelanica (Summer)	48.3 ± 1.91 [c]	40.7 ± 5.89 [b]	0.85 ± 0.14 [c]	3.02 ± 0.12 [b]

Table 2. Mitochondrial parameters from brachiopod adductor muscle in relative and absolute terms. Dissimilar letters indicate significant differences ($p < 0.05$). Mean ± SE. $N = 8$ for each group.

	$Vv_{(mt,f)}$	Mitochondrial Volume (μm^3)	$Sv_{(im,mt)}$ (μm^2 μm^{-3})	Cristae Surface Area (μm^2)	Aerobic Capacity (μm^2 μm^{-3})
L. uva	0.013 ± 0.0010^{a}	0.84 ± 0.043^{a}	25.9 ± 1.32^{a}	21.5 ± 1.43^{a}	0.34 ± 0.028
L. neozelanica (Winter)	$0.009 \pm 0.0007^{a,b}$	$0.51 \pm 0.082^{a,b}$	35.1 ± 1.99^{b}	18.0 ± 3.11^{a}	0.33 ± 0.030
L. neozelanica (Summer)	0.009 ± 0.0016^{b}	0.26 ± 0.025^{b}	26.7 ± 1.74^{a}	6.96 ± 0.78^{b}	0.23 ± 0.042

Figure 1 A and B

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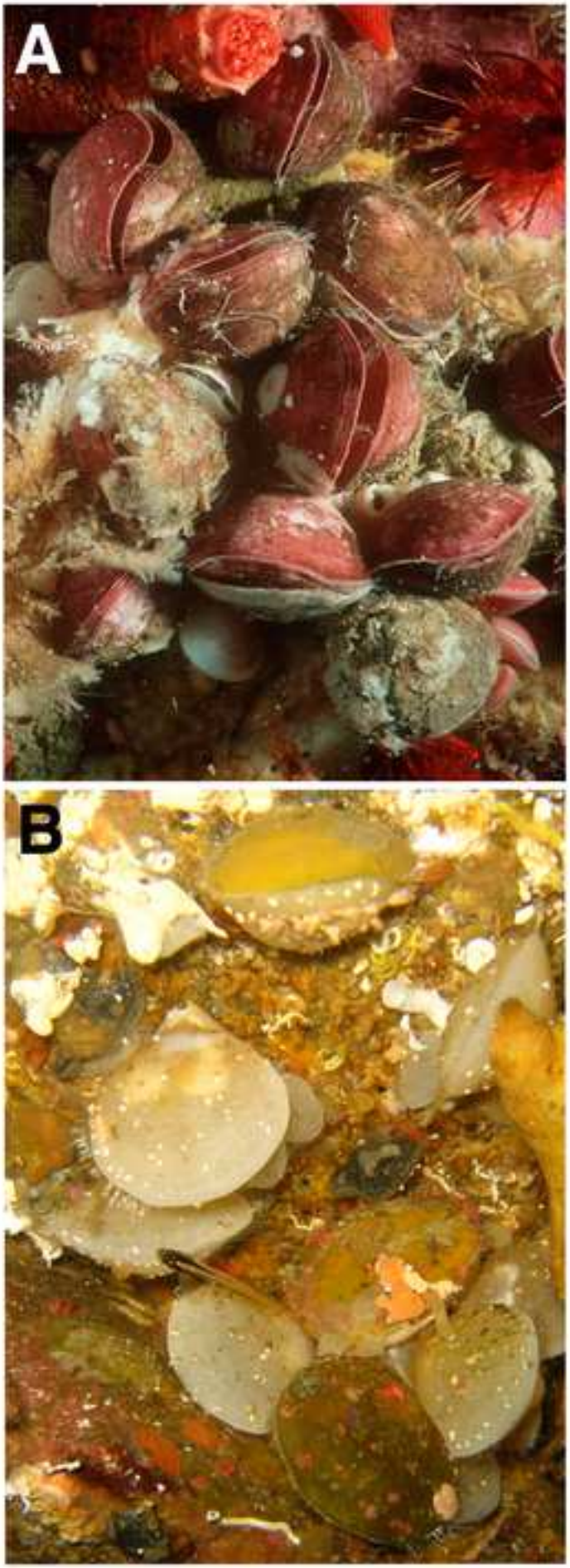

Supplemental Figure 2 A and B

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