Mitochondrial dynamics underlying thermal plasticity of cuttlefish (*Sepia officinalis*) hearts

Key words: Temperature sensitivity, cephalopod, evolutionary adaptation, thermal acclimation, proton leak, lactate and octopine dehydrogenase, respiration, systemic and branchial hearts, cardiac fibres

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ABSTRACT

In the eurythermal cuttlefish *Sepia officinalis*, performance depends on hearts that ensure systemic oxygen supply over a broad range of temperatures. We therefore aimed to identify adjustments in energetic cardiac capacity and underlying mitochondrial function supporting thermal acclimation and adaptation that could be critical for the cuttlefish's competitive success in variable environments. Two genetically distinct cuttlefish populations were acclimated to 11°C, 16°C and 21°C, respectively. Subsequently, skinned and permeabilised heart fibres were used to assess mitochondrial functioning by means of high-resolution respirometry and a substrate-inhibitor protocol, followed by measurements of cardiac citrate synthase and cytosolic enzyme activities. Temperate English Channel cuttlefish had lower mitochondrial capacities but larger hearts than subtropical Adriatic cuttlefish. Warm acclimation to 21°C decreased mitochondrial complex I activity in Adriatic cuttlefish and increased complex IV activity in English Channel cuttlefish. However, compensation of mitochondrial capacities did not occur during cold acclimation to 11°C. In systemic hearts, thermal sensitivity of mitochondrial substrate oxidation was high for proline and pyruvate but low for succinate. Oxygen efficiency of catabolism rose from 11°C to 21°C via shifts to oxygen-conserving oxidation of proline and pyruvate and via reduced relative proton leak. The changes observed for substrate oxidation, mitochondrial complexes, relative proton leak or heart weights improve energetic efficiency and essentially seem to extend tolerance to high temperatures and reduce associated tissue hypoxia. We conclude that cuttlefish sustain cardiac performance and thus, systemic oxygen delivery over short and long-term changes of temperature and environmental conditions by multiple adjustments in cellular and mitochondrial energetics.

INTRODUCTION

The evolution of modern cephalopods was shaped by the rising competition with marine vertebrates and led to innovations that advance high levels of performance. Concomitant increases of energy demands required extant cephalopods to optimise supply and use of oxygen (O'Dor and Webber, 1986; O'Dor and Webber, 1991). As a result, cephalopods evolved high concentrations of blood pigment optimised for oxygen transport and a closed circulatory system driven by two branchial and one powerful systemic heart (Schipp, 1987; Wells and Smith, 1987; Wells, 1992; Pörtner and Zielinski, 1998). However, design constraints were involved, leading to locomotion by jet propulsion and comparatively low blood oxygen carrying capacities. Optimisation of performance led at least some cephalopod species to operate at their functional limits (O'Dor and Webber, 1986; Pörtner, 2002b). Environmental stressors such as fluctuations in ambient temperature or ambient hypoxia may be particularly challenging for the highly oxygen dependent cephalopods that face high competitive pressure (Rosa and Seibel, 2008).

Body functions of animals operate only within a certain thermal range and are set by ambient temperature for ectotherms. According to recent evidence, oxygen supply becomes limiting at high temperatures when oxygen demand increases due to limited functional capacities of the circulation and ventilation system to deliver oxygen (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). Adaptive adjustments of e.g. ventilatory musculature and heart rate or stroke volume are suitable to compensate for temperature induced impairments of oxygen supply (Wells, 1992; Frederich and Pörtner, 2000). These adjustments, however, involve changes in the capacity of mitochondria to provide sufficient aerobic energy to vital tissues like the heart (Pörtner, 2002a). Low temperatures also cause capacity limitations, which may involve insufficient release of oxygen to tissues by the blood pigment haemocyanin (Melzner et al., 2007b) and limited mitochondrial energy provision as required to power circulation or ventilation (Pörtner, 2002a).

Ectothermic hearts play a major role in defining limits of aerobic performance and thermal tolerance, as demonstrated by impaired cardiac function close to extreme temperatures or exercise levels (Farrell, 2002; Somero, 2010). In cephalopods, systemic hearts cover increased metabolic demands during either exercise or rising temperatures by a 2-3 fold increase of stroke volume or heartbeat frequency (Wells, 1992), but show limited performance at high critical temperatures, as indicated in *Sepia officinalis*, where blood perfusion fails to increase further beyond 23°C (Melzner et al., 2007a; Melzner et al., 2007b).

Yet, unlike in fishes, cephalopod systemic hearts receive well oxygenated blood and gain support by two accessory hearts, contractile blood vessels and mantle pressure oscillations (Schipp, 1987; Melzner et al., 2007a). Adversely, high blood viscosity, 2-3 fold lower blood oxygen carrying capacities and higher resting metabolic rates compared to haemoglobin bearing fish with a similar lifestyle (Wells, 1992; Pörtner, 1994) demand higher performance of the systemic heart. While cephalopod hearts are able to meet this workload within limits during acute rises of metabolic demand, it is unknown whether they adjust to seasonal (i.e. acclimation) or long term environmental changes over multiple generations (i.e. evolutionary adaptation). Such temperature acclimation or adaptation has been shown for fish hearts. Here, changes comprise increases in heart size (Goolish, 1987; Kent et al., 1988), increased mitochondrial content (Kleckner and Sidell, 1985; Johnston and Harrison, 1987; Kolok, 1991), shifts from carbohydrate to fatty acid oxidation (Sephton and Driedzic, 1991; Sidell et al., 1995) or enhanced activities of enzymes essential in aerobic metabolism (Crockett and Sidell, 1990; Podrabsky et al., 2000), following cold-exposure. Yet, evidence for such temperature related adjustments is still lacking for cephalopod hearts.

The eurythermal common cuttlefish *Sepia officinalis* (Linnaeus 1758) lives on the continental shelf from the cold-temperate eastern North Atlantic to warm-subtropical Mediterranean and Atlantic waters off the Senegalese coast and follows a bottom dwelling, migratory life style on rocky to sandy grounds down to 200 m depth. Cuttlefish are rather sluggish, grow fast (up to two kg) and spawn after one or two years in shallow waters and die thereafter (Jereb and Roper, 2005). In this study, we compared a cuttlefish population from the English Channel living between 9°C and 17.5°C with a genetically distinct Mediterranean population (Wolfram et al., 2006) facing a range from 10°C to 25°C, respectively, between winter and summer (Boucaud-Camou and Boismery, 1991; Artegiani et al., 1997; Wang et al., 2003).

In this study, we aimed to understand how temperature changes affect the heart of the common cuttlefish *Sepia officinalis* by (1) exploring cardiac adjustments following long term genetic isolation in a temperate and subtropical habitat as well as (2) following thermal acclimation and (3) by investigating the thermal sensitivity of heart mitochondria.

MATERIAL AND METHODS

Experimental animals

Wild laid eggs of the European cuttlefish (*Sepia officinalis*) were collected from the temperate Oosterschelde lagoon, Netherlands (3°56′E, 51°35′N) and the subtropical Venetian lagoon close to Chioggia, Adria, Italy (12°18′E, 45°13′N). Populations from these localities form geographically distanced and genetically distinct clades without evidence of on-going genetic exchange (Wolfram et al., 2006; Perez-Losada et al., 2007). Thus, inter-population differences will most likely reflect evolutionary adaptation or genetic drift. About 300 eggs from five to six different egg masses (50 to 60 eggs per mass) were collected from a frequently visited spawning ground (>100 individuals per day) in the Oosterschelde lagoon and 200 eggs from more than ten egg masses were collected by scuba diving and snorkelling in the Venetian lagoon in 2008 and 2009. After transport to our institute individuals from each population were hatched and raised in separate tanks, connected to a re-circulating aquaculture system at constant temperature (mean ± s.d., 15.9°C ± 0.1) at suitable stocking densities (Hanley et al., 1998) under constant 12 h dark:12 h light cycle. Hatchlings were fed daily with live shrimps (*Neomysis integer*, *Palaemonetes varians* and *Crangon crangon*) and after reaching a bigger size, exclusively with frozen brown shrimp (*Crangon crangon*). Water quality parameters were monitored weekly and kept at levels appropriate for cuttlefish culture (Hanley et al., 1998) (mean ± s.d., O_2 = 10.07 ± 0.43 mg l^{-1}, pH = 8.05 ± 0.02, salinity = 32.6 psu ± 0.5, NH_4^+ < 0.2 mg l^{-1} , NO_2^- < 0.2 mg l^{-1}, NO_3^- < 80 mg l^{-1}) by means of water treatment (protein skimmers, mechanical and biological filters, UV sterilisation) or water replacement.

After animal size had reached 30 - 40 g, each population was divided into three groups that were acclimated to 11°C, 16°C and 21°C from 8 to 22 weeks. These acclimation temperatures were selected to expose European cuttlefish to their seasonal and depth dependent temperature range, 10°C to 17.5°C for the English Channel population and 10°C to 25°C for the Adriatic Sea population. Each group was kept in separate tanks connected to independent re-circulating systems for each temperature. Only animals showing normal behaviour without signs of illness (skin infections mostly due to jetting against tank walls) were selected for experiments. Although Adriatic cuttlefish weighed significantly more in the 21°C group (Supplementary table S2) at the end of the acclimation period (possibly due to

enhanced growth rates), correlation analysis did not show clear evidence that weight differences obscured our data.

Mitochondrial respiration

Dissection and muscle fibre preparation

Animals were anesthetized in 3% ethanol until non-responsive before culling. Subsequently, total weight, mantle length, total length and sex were recorded. After opening the mantle cavity, samples of blood, gills and ink were taken and frozen for further experiments. The three hearts were excised starting with the branchial hearts and placed immediately into 1 ml ice-cold biopsy buffer (modified after (Kuznetsov et al., 2008)) to preserve mitochondrial function. The biopsy buffer contained (in mmol l^{-1}, 2.77 CaK_2EGTA, 7.23 EGTA, 14.46 KOH, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 20 imidazole, 0.5 dithiothreitol (DTT), 50 MES, 588 sucrose, 252 glycine, pH 7.4 at 26°C, 1000 mosmol l^{-1}).

After removal of non-cardiac tissues and weighing of all three hearts, approximately 50 mg of systemic heart tissue were placed into a drop of biopsy buffer on a Petri dish on ice and then coarsely torn apart and dissected into small fibre bundles using two tweezers. The remainder of heart tissue and the branchial hearts were snap-frozen in liquid nitrogen and stored at -80°C for subsequent enzyme assays. Fibre bundles were immediately transferred to 1 ml biopsy buffer in a 12 well multiwell culture plate and permeabilised with 50 µg ml^{-1} saponin (Note: preliminary testing confirmed appropriate saponin concentration) by gentle mixing (115 rpm) on ice for 30 min. The fibres were then removed and washed three times for 10 min in 2 ml ice-cold mitochondrial respiration medium (in mmol l^{-1}, 50 HEPES, 25 KH_2PO_4, 0.5 EGTA, 50 KCl, 50 NaCl, 10 $MgCl_2$, 20 taurine, 50 lactobionate, 350 sucrose, 150 glycine, 1 g l^{-1} freshly added fatty acid free BSA, pH 7.4 at 22°C, 1000 mosmol l^{-1}, modified after (Mommsen and Hochachka, 1981; Agnisola et al., 1991; Kuznetsov et al., 2008)) and stored in ice-cold respiration medium until use (protocol modified after (Saks et al., 1998)).

Measurements of mitochondrial respiration

Before each assay, fibres were blotted dry on chilled Whatman® paper, divided into two 2-6 mg bundles and transferred to 2 ml duplicate chambers of an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria) containing air saturated respiration medium at the experimental temperature. Mitochondrial respiration was then measured online as background corrected weight specific oxygen consumption rate (pmol O_2 s^{-1} mg^{-1}, i.e. negative time

derivative of oxygen concentration) using DatLab analysis software (Oroboros Instruments, Innsbruck, Austria). Assays were performed consecutively at 11°C, 16°C and 21°C in randomised order. Chambers were washed with 96% ethanol and Milli-Q water to thoroughly remove inhibitors and substrates after each assay. To protect fibres from extreme oxygen levels, oxygen concentrations in chambers were kept between 80 nmol O_2 ml^{-1} (maximum stimulated respiration remained stable down to 80 nmol O_2 ml^{-1}) and air saturation (270-340 nmol O_2 ml^{-1}) by re-oxygenation with pure oxygen gas.

To assess mitochondrial function, respirometry on permeabilised skinned heart fibres was combined with a substrate-inhibitor protocol as follows. Mitochondria were first fuelled successively and in excess with the amino acid proline (5 mmol l^{-1}), ADP (2.5 mmol l^{-1}) as well as pyruvate (5 mmol l^{-1}) and succinate (10 mmol l^{-1}) in order to reach maximum coupled oxidative phosphorylation (i.e. state 3 respiration tied to ATP production). The choice of substrates corresponds to own tests and to previous studies that showed high rates of oxidation for proline, pyruvate and succinate in squid hearts (Ballantyne et al., 1981; Mommsen and Hochachka, 1981). In situ, cephalopod hearts derive pyruvate most evidently from blood glucose or oxidised octopine and proline from high intracellular stores (> 12 µmol g^{-1}). Succinate can originate from both pyruvate derivatives and amino acids (e.g. ornithine, arginine, glutamate and proline) with the latter feeding into the Krebs cycle at the level of alpha-ketoglutarate (Ballantyne et al., 1981; Mommsen and Hochachka, 1981; Hochachka and Fields, 1982).

Integrity of mitochondrial membranes was tested by the addition of cytochrome c (0.01 mmol l^{-1}) that would stimulate respiration in case of damaged outer membranes. Proline stimulated respiration without ADP (state 2) provided an estimate of mitochondrial proton leak (Iftikar et al., 2010). Uncoupling of respiration from ATP production by carbonylcyanide-p-(trifluoromethyl) phenylhydrazone (FCCP, up to 2.5 µmol l^{-1}) denoted maximum capacity of the electron transport chain. The loss of activity with rotenone (2.5 µmol l^{-1}) indicated NADH dehydrogenase (complex I) activity. The loss of activity with antimycin A (2.5 µmol l^{-1}) quantified the non-mitochondrial background respiration. Cytochrome c oxidase (complex IV) activity was tested using the redox pair ascorbate (2 mmol l^{-1}) and N,N,N',N'- tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mmol l^{-1}, see list in supplementary table S1). Background auto-oxidation of the redox pair was determined for all experimental temperatures and subtracted from final results. Suitability and concentrations of substrates were tested beforehand. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany)

Enzyme assays

Protein extraction and sample preparation

To extract the protein, frozen heart tissue was ground by hand in a mortar filled with liquid nitrogen. The frozen tissue powder was then weighed and suspended in 10 volumes (w:v) of ice cold extraction buffer (50 mmol l^{-1} Tris-HCl (pH 7.4 at 16°C), 1 mmol l^{-1} EDTA, 0.1% Triton X-100) and sonicated for 90 s at 0°C in a Branson Sonifier 450 (output control 8, Duty cycle 50%). Following two centrifugations for 10 min at 6000g and 4°C, supernatants were used for protein and enzyme assays. Pellets remaining after centrifugation were resuspended and re-extracted in ice cold extraction buffer to test for residual protein and citrate synthase activity.

For enzyme assays, samples were diluted 1:10 (v:v) with 75 mmol l^{-1} Tris-HCl (pH 8.1 at 16°C) and equilibrated to approximately 16°C using a temperature controlled metal block connected to a thermostat (Haake C25, Thermo Scientific, Karlsruhe, Germany) prior to each measurement. Absorbance was measured in triplicates using 96 well F-bottom microplates (Nunc GmbH & Co. KG, Wiesbaden, Germany), a multiplate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany) and analyzing software (FLUO 32, version 4.31 R5).

Enzyme activities and protein content

Citrate synthase (EC 4.1.3.7) activity was determined after Sidell et al. (1987) as the increase of absorbance at 412 nm by means of DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)). First, background deacylase activity was measured with 20 µl of diluted sample supernatant and 160 µl reaction mixture (in mmol l^{-1}, 75 Tris-HCl, 0.25 DTNB, 0.4 acetyl~CoA, pH 8.1) followed by the addition of 20 µl 0.5 mmol l^{-1} oxaloacetate to start the reaction. As DTNB reacts with sulphur groups (SH), dithiothreitol (DTT) was used as standard for the calculation of SH group turn-over.

Activities of octopine dehydrogenase (ODH, EC 1.5.1.11) and lactate dehydrogenase (LDH, EC 1.1.1.27) indicate capacities to form or recycle anaerobic end products in cephalopods (Grieshaber and Gäde, 1976; Storey, 1977; Storey and Storey, 1979). ODH and LDH activities were determined after Storey (1977) and Driedzic et al. (1990) as the decrease of absorbance at 340 nm. First, background activity was measured by combining 20 µl of diluted sample supernatant and 160 µl reaction mixture (in mmol l^{-1}, 100 Tris-HCl (pH 7.0), 0.45 NADH, 1 KCN, 10 arginine were added for quantifying the ODH reaction) followed by the addition of 20 µl 4 mmol l^{-1} pyruvate to start the reaction. Final ODH activity was

determined by subtracting the LDH activity, which was measured simultaneously. NADH was used as a standard.

Enzyme activities were standardised to protein content determined after Bradford (1976). Sample supernatant and pellet homogenate were diluted 1:10 (v:v) with 0.9% NaCl. 5 µl were transferred to 250 µl Bradford dye reagent (0.1 mg ml^{-1} Coomassie Brilliant Blue G-250, 5% ethanol, 8.5% H_3PO_4). After mixing and incubation for 10 min at room temperature (21°C), absorbance was recorded at 595 nm. Bovine albumin serum (BSA) was used as protein standard.

Statistical analysis

Statistical analysis was performed to spot significant differences ($P < 0.05$) using SPSS (SPSS© Inc., version 14.0.1) by employing tests as followed: analysis of variance (ANOVA) or non-parametric tests; additional post-hoc Tukey or Hochberg's GT2 test for equal or unequal sample sizes respectively to compare acclimation treatments or assay temperatures; Pearson's correlation analysis to test for allometric effects; Kolmogorov-Smirnov and Levene's test assessed normality and homogeneity of variances, respectively. Data were expressed as means and the range of their 95% confidence interval if not stated otherwise. Outliers were detected using Nalimov's test ($P < 0.01$) and excluded if justified. The temperature coefficient Q_{10} was calculated as follows:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{(T_2 - T_1)}}$$

R denotes the respiratory rate at a higher (T_2) or lower temperature (T_1). Mitochondrial respiration was expressed per mg wet weight of blotted heart fibres.

RESULTS

Evolutionary Adaptation

Mitochondrial respiration differed clearly between the two genetically distinct cuttlefish populations. On average, cardiac mitochondria, fully fuelled by substrates and ADP (state 3), showed significantly higher respiration rates in Adriatic cuttlefish with 41.1 pmol O_2 s^{-1} mg^{-1} (95% CI range from 37.3 to 44.9 pmol O_2 s^{-1} mg^{-1}) compared to only 32.9 pmol O_2 s^{-1} mg^{-1} (30.0-35.9) as seen in English Channel cuttlefish (ANOVA, $F(1, 128) = 11.92$, $P < 0.01$, calculations based on entire pooled data set). These 20% higher cardiac aerobic capacities in cuttlefish from warmer waters were most pronounced at lower assay temperatures, and were mainly due to a higher contribution of pyruvate to overall respiration (average contribution of 15.2 pmol O_2 s^{-1} mg^{-1}, (13.1-17.3)) for Adriatic cuttlefish compared to 9.0 pmol O_2 s^{-1} mg^{-1}, (7.8-10.2) for English Channel cuttlefish, Figure 1). Consequently, fractions of pyruvate dependent respiration constituted 35.4% (32.8-38.0) in systemic hearts of Adriatic Sea but only 26.2% (24.1-28.3) in English Channel cuttlefish. Conversely, proline dependent fractions were higher (45.2% (42.5-47.8)) in English Channel- than in Adriatic Sea cuttlefish (35.2% (32.4-38.0)). Succinate dependent fractions were similar in the two populations.

While body weights did not differ between populations (ANOVA, F (1, 64) = 2.36, P = 0.13), English Channel cuttlefish contained 43% heavier systemic and 38% heavier branchial hearts than Adriatic cuttlefish (systemic hearts: Kruskal-Wallis, $H(1) = 13.69$, $P < 0.01$; branchial hearts: Kruskal-Wallis, $H(1) = 10.22$, $P < 0.01$, for raw data see supplementary table S2). Relative weights of systemic hearts constituted 0.083% (0.078-0.088) in English Channel and 0.059% (0.056-0.062) in Adriatic cuttlefish. Similarly, relative weights of branchial hearts constituted 0.047% (0.044-0.050) in English Channel and 0.039% (0.036-0.042) in Adriatic cuttlefish. As a result, respiration calculated for the whole systemic heart (state 3 respiration x heart weight) did not differ between populations (ANOVA, F (1, 41) = 0.03, P = 0.86), due to the larger hearts in English Channel cuttlefish.

Further, systemic hearts of Adriatic cuttlefish showed partly higher protein specific enzyme activities compared to English Channel cuttlefish, which was most apparent for octopine dehydrogenase (Table 1). Enzyme activities of branchial hearts were similar between populations except for enhanced lactate dehydrogenase activities in English Channel cuttlefish acclimated to 11°C and 16°C (Table 1).

Thermal acclimation

Acclimation of cuttlefish to 11°C, 16°C and 21°C did not affect overall maximum state 3 respiration in the English Channel (ANOVA, $F(2, 68) = 0.04$, $P = 0.96$) and in the Adriatic Sea populations (Kruskal-Wallis, $H(2) = 0.94$, $P = 0.63$) but caused minor shifts in substrate dependent fractions (Figure 1) and slight changes in cardiac enzyme activities of English Channel cuttlefish, shown by enhanced citrate synthase activities at 11°C and lower lactate dehydrogenase activities at 21°C compared to animals acclimated to 16°C (Table 1). Also, relative heart weights did not change with thermal acclimation for systemic- (ANOVA, English Channel F (2, 29) = 0.50, $P = 0.61$, Adriatic Sea F (2, 30) = 0.95, $P = 0.40$) and branchial hearts (ANOVA, English Channel F (2, 29) = 0.46, $P = 0.64$, Adriatic Sea F (2, 30) = 3.0, $P = 0.07$).

Thermal acclimation affected the activity of mitochondrial complexes in systemic heart fibres of cuttlefish. In this regard, Adriatic cuttlefish acclimated to 21°C showed between 10-19% lower complex I activity compared to cuttlefish acclimated to 11°C and 16°C whereas complex I activity in English Channel cuttlefish did not respond to thermal acclimation (Figure 2A). In contrast, systemic hearts of English Channel cuttlefish contained mitochondria that displayed increased complex IV activity following acclimation to 21°C, at assay temperatures of 11°C and 21°C (Figure 2B). In Adriatic cuttlefish hearts, however, complex IV activity remained unaffected by thermal acclimation but showed between 8-30% higher enzyme activity at lower acclimation temperatures compared to English Channel cuttlefish (Figure 2B).

Thermal sensitivity of cardiac mitochondria

In both cuttlefish populations cardiac mitochondria responded similarly to acute temperature changes ranging from 11°C to 21°C. State 3 respiration increased from 20.4 (18.1-22.8) to 46.4 pmol O_2 s^{-1} mg^{-1} (42.9-49.9) in English Channel and from 27.8 (24.8-30.8) to 51.2 pmol O_2 s^{-1} mg^{-1} (45.3-57.1) in Adriatic cuttlefish, with average Q_{10} of 2.3 in English Channel and 2.0 in Adriatic animals (Figure 3). Respiration resolved for substrates revealed a high thermal sensitivity of proline- and pyruvate stimulated respiration but a low thermal sensitivity of succinate stimulated respiration (Figure 3). This caused succinate to contribute most to overall state 3 respiration at 11°C assay temperature (English Channel 40.4% (37.8-43.0); Adriatic Sea 43.4% (39.6-47.2)) but to be a minor substrate at 21°C (English Channel 18.7% (15.9-21.5); Adriatic Sea 19.0% (16.0-22.0)). Conversely, proline and pyruvate

prevailed as oxidative substrates at 21°C assay temperature, whereas their fractions declined upon cooling towards 11°C (Figure 1).

Mitochondrial complexes displayed different temperature dependencies upon acute exposure. Similar to succinate stimulated respiration (which indicates activity of complex II) the activity of complex IV (indicated by respiration fuelled with ascorbate and TMPD) showed low thermal sensitivity (Figure 3).

Interestingly, although absolute leak rates increased with warmer assay temperatures up to 21°C, mitochondrial proton leak relative to ADP stimulated respiration decreased in systemic hearts of English Channel cuttlefish and by trend in Adriatic cuttlefish upon warming (Figure 4A, B). Outer membrane integrity, tested by cytochrome c addition, decreased with increasing assay temperatures in both cuttlefish populations (Figure 5). Further, English Channel cuttlefish showed more defective outer membranes and higher relative proton leak at 11°C assay temperature compared to Adriatic cuttlefish (Figure 5).

DISCUSSION

Evolutionary adaptation

There is strong evidence for evolutionary changes of heart function between the genetically distinct temperate and subtropical cuttlefish populations. Systemic hearts of temperate (English Channel) cuttlefish contained lower aerobic and partly lower anaerobic capacities (Figure 1 & Table 1) that may reduce their cardiac output and hence lower their ability to increase heart rates during exercise or elevated temperatures. Whether mitochondrial density parameters, enzyme numbers or specific enzyme activities accounted for the differential aerobic capacities remains to be resolved. Nevertheless, similar total heart respiration between populations showed that English Channel cuttlefish compensate for reduced respiratory capacities by larger hearts. Compensatory increases of heart weight are also common in teleost fish living at cooler temperatures (Foster et al., 1993; Driedzic et al., 1996). Larger hearts support pumping of larger blood volumes per stroke, thereby, they may compensate for low energetic capacities but also for rising blood viscosities at colder temperatures (Goolish, 1987; Driedzic et al., 1996). Thus, cuttlefish heart function in the cold may rather be sustained through an increase in organ size, than through an increase of cellular energetic capacities.

Furthermore, the findings of predominant oxidation of proline in systemic hearts of temperate cuttlefish but enhanced pyruvate oxidation in subtropical cuttlefish are well in line with findings for cold adapted fish that show suppressed carbohydrate metabolism but enhanced lipid oxidation (Crockett and Sidell, 1990; Sidell et al., 1995). Fuels like lipids and proline are less oxygen efficient than carbohydrates, which are generally favoured in cephalopods (Hochachka, 1994). Carbohydrate oxidation reduces oxygen consumption of the heart and threats from tissue hypoxia due to higher ATP yields per molecule of oxygen consumed (Higgins et al., 1980; Kahles et al., 1982; Hochachka, 1994). In cephalopod hearts, a mole of proline requires half a mole of dioxygen to oxidize it to glutamate before entering the Krebs cycle, thus decreasing its oxygen efficiency (Mommsen and Hochachka, 1981). Therefore, impaired oxygen supply at higher temperatures or environmental hypoxia, which cuttlefish face often in northern Adriatic lagoons (Diaz, 2001; Sorokin and Dallocchio, 2008), may favour carbohydrates and thus, pyruvate as the more oxygen efficient substrate.

Thermal acclimation

Substrate oxidation

In cuttlefish hearts, thermal acclimation did not affect aerobic capacities (i.e. state 3 respiration, Figure 1) and caused only minor changes of citrate synthase and lactate dehydrogenase activities in temperate cuttlefish (Table 1). At first glance, this contrasts with the thermal compensation found for English Channel cuttlefish. Here, routine metabolic rates of cuttlefish acclimated to 20°C fell below routine metabolic rates of individuals acclimated to 15°C, once above 20°C experimental temperature, thereby supporting an upward shift of limiting temperatures beyond 23°C (Melzner, 2005). This pattern of thermal acclimation may well be explained by a suppression of oxygen consumption rates in organs other than the hearts (e.g. hepatopancreas, mantle). Such a one-sided reduction of oxygen consumption in some tissues but concomitant maintenance of cardiac capacities may then free aerobic scope necessary to shift thermal tolerance upwards. On the other hand, in parallel to cardiac capacities, routine metabolic rates decline by 2-3.5 times from 20°C to 8°C to equally low levels irrespective of the acclimation mode (Melzner, 2005). As a result, cardiac as well as whole animal energetic capacities decline with decreasing temperatures and thus match a reduction of energy turnover during the cold season, marked by reduced growth rates of cuttlefish during winter (Le Goff and Daguzan, 1991).

Mitochondrial complexes

Effects of warm acclimation on mitochondrial complexes I and IV in systemic hearts of cuttlefish suggest modifications that delay heat-induced tissue hypoxia and formation of reactive oxygen species (ROS). Due to constraints on their systemic (i.e. low blood oxygen carrying capacity) and intracellular oxygen transport system (e.g. lack of intracellular oxygen delivering protein like myoglobin, (Hochachka, 1994)), cephalopods are chronically prone to facing critically low intracellular oxygen concentrations, particularly when high temperatures induce tissue hypoxia (Pörtner, 2001). Gnaiger et al. (1998) argued that an excess capacity of complex IV sustains high affinities for oxygen in mitochondria. Therefore, the increase of complex IV capacity in systemic hearts of English Channel cuttlefish following warm acclimation to 21°C (Figure 2B) likely enhanced oxygen affinity, thus supporting oxygen diffusion to mitochondria at high and hypoxia inducing temperatures. This response is in agreement with fishes that are capable to increase cardiac complex IV activity following warm-acclimation as seen in the highly hypoxia tolerant carp (Cai and Adelman, 1990) but also in cod (Foster et al., 1993) or in the liver of Antarctic eelpout (Windisch et al., 2011).

ROS formation in response to heat stress and environmental hypoxia is common among marine ectotherms (Abele et al., 2007), and may occur more often in the warm and often hypoxic lagoons of the northern Adriatic Sea during summer (Diaz, 2001; Sorokin et al., 2002) where cuttlefish are common (Rossetto, 2001). Complex I is one of the major sites of ROS production and has been related to cardiac failure (Ide et al., 1999; Sorescu and Griendling, 2002), therefore, a suppression of complex I capacity following warm-acclimation in Adriatic cuttlefish (Figure 2A) may reduce heat and hypoxia related ROS formation to preserve mitochondrial function. As a corollary, changed activities of mitochondrial complexes following warm acclimation may serve cuttlefish to reduce temperature induced hypoxia or harmful oxygen stress in cardiac tissues.

Thermal sensitivity of cardiac mitochondria

Substrate oxidation

During summer, cuttlefish pass a steep thermocline during their daily vertical migration with possible temperature changes of up to 2°C per m (e.g. Adriatic Sea: 23°C to 14°C down to 80 m; English Channel: 19°C to 11°C down to 40 m) (Artegiani et al., 1997; Sharples et al., 2001). Our findings demonstrate that noticeable increases of mitochondrial respiration (state 3) from 11°C to 21°C support systemic hearts of cuttlefish to operate aerobically over this range of temperatures. Interestingly, various substrates contributed differently to this thermal response (Figure 3). The substrate dependent effect on thermal sensitivity of oxidative pathways may allow mitochondria to produce aerobic energy over a broader range of temperatures. While less temperature sensitive pathways (e.g. that augment succinate) attenuate a rapid decline of mitochondrial respiration (i.e. ATP provision) at low temperatures, thermally more sensitive pathways (e.g. fuelled by pyruvate or proline) complement less responsive pathways at higher temperatures to match enhanced ATP demands of the heart. Although pyruvate and proline oxidation declines at low temperatures, *in situ* supply of succinate may be sustained by amino acids such as glutamate, ornithine or arginine that are well oxidized and readily available from the blood or intracellular stores (Ballantyne et al., 1981; Mommsen and Hochachka, 1981; Hochachka and Fields, 1982; Mommsen et al., 1983). Observations in fish hearts support this pattern, as fatty acid oxidation has mostly lower or at least different thermal sensitivities than carbohydrate oxidation, which kicks in in the warmth (Sephton et al., 1990; Sephton and Driedzic, 1991).

The predominance of pyruvate and proline oxidation at higher temperatures (Figure 1) improves oxygen efficient production of ATP and likely reduces the threat of tissue hypoxia.

After entering mitochondria, proline is oxidized to glutamate and then enters the Krebs cycle via alpha-ketoglutarate and yields 4-5 NADH and 1 $FADH_2$ (i.e. assuming no diffusive loss of products), similar to pyruvate that yields 4 NADH and 1 $FADH_2$ during its oxidation via the citric acid cycle (Storey and Storey, 1983). As a consequence, proline and pyruvate feed about 80% of their electrons into complex I and thus produce more ATP per mole of oxygen consumed. In contrast, succinate yields 1 NADH and 1 $FADH_2$ and thus, diverts only 50% of its electrons to complex I when fully catabolized to oxaloacetate. Oxygen efficient ATP production may be advantageous at high temperatures when oxygen becomes limiting ((Pörtner, 2010) for recent review) and is well in line with the general trend of modifications in substrate use by mitochondria that enhance oxygen efficiency in cephalopods (Hochachka, 1994).

Membrane leakiness and integrity

Surprisingly, in cuttlefish hearts, relative proton leak decreased and outer membrane integrity increased (i.e. shown by decreased stimulation of respiration by cytochrome c) up to a temperature close to the whole animal critical temperature of 23°C ((Melzner et al., 2007b), Figure 4, 5). In contrast to the present observation, relative proton leak increases with temperature in mitochondria isolated from hearts, red muscle and liver of fish (Hardewig et al., 1999; Fangue et al., 2009; Hilton et al., 2010), gills of Antarctic bivalves (Pörtner et al., 1999) or the body wall of a lugworm (Sommer and Pörtner, 2002). Our finding thus contrasts with the general view that higher temperatures increase membrane fluidity and thus proton leak (Hazel, 1995; Pörtner, 2001). This could be explained by a decrease of mitochondrial membrane potential via increased ATP synthase activity relative to electron flux through the electron transport system, which would induce reduced proton gradients over the membrane leading to reduced proton leak (Nicholls, 2004). Alternatively, structural changes of mitochondrial membranes that reduce membrane permeability may have occurred, as indicated by reduced respiratory stimulation by cytochrome c (Figure 5), which passes the outer membrane by diffusion only (Gellerich et al., 2000). Such evident changes of the outer membrane permeability likely affected the inner mitochondrial membrane too and hence supported the observed decline of relative proton leak with rising temperatures. Even though the underlying mechanisms remain unclear, decreasing relative proton leak with acutely rising temperature aids cuttlefish hearts to be more oxygen efficient and thereby shift temperatures that entail oxygen deficiency to higher tolerated values. These functional characteristics may be adaptive in supporting eurythermy of this species.

Interestingly, temperate English Channel cuttlefish mitochondria displayed lower respiratory capacities and contained more permeable and therefore less efficient membranes at 11°C assay temperature than the subtropical Adriatic cuttlefish (Figure 4, 5). Their mitochondria operate thus less efficient and more "costly" at cool temperatures. Conversely, a higher "futile cycling" through proton leak would make them more responsive to sudden increases in workloads. This may be needed less in warm acclimated hearts.

Conclusion

Our study about cuttlefish heart fibres and their mitochondria revealed inherent potential to cope with thermal challenges faced during an individual's lifetime, but also genetic plasticity between populations relevant for adaptation to long-term environmental change. Most modifications improve cardiac efficiency and extend tolerance to high temperatures and associated hypoxemic conditions. Cold compensation on evolutionary time scales occurs via a shift in substrate and enhanced proton leak as a sign of enhanced futile cycling in mitochondrial metabolism. Lack of cold compensation by adjusting mitochondrial or enzyme capacities during cold acclimation, on the other hand, suggest decreased cardiac energetic capacities during the cold season. Overall, we conclude that the observed flexibility of cardiac function, based on specific cephalopod-type characteristics, assures cardiac power output and systemic oxygen delivery at various temperatures. It thus conforms to the capacity of *Sepia officinalis* to tolerate a broad range of temperatures and supports their ability to contend with fishes - their prime competitors - in rapidly changing environments.

LIST OF SYMBOLS AND ABBREVIATIONS

ATP	Adenosin-5'-triphosphate
BSA	Bovine Serum Albumin
Complex I	NADH dehydrogenase
Complex IV	Cytochrome c oxidase
DTNB	5,5'-dithio-bis-(2-nitro-benzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid
LDH	Lactate dehydrogenase
ODH	Octopine dehydrogenase
Q_{10}	Temperature coefficient
ROS	Reactive oxygen species
TMPD	N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride

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FIGURES LEGENDS

Figure 1: Maximum state 3 respiration (pmol O_2 s^{-1} mg^{-1}) resolved for each substrate, after addition of ADP, at 11°C, 16°C and 21°C assay temperature in comparison between acclimation treatments (*N* = 5-11) as well as between cuttlefish from (A) the English Channel and (B) Adriatic Sea. Significant differences ($P < 0.05$) between acclimation groups are marked by asterisks and for total state 3 respiration between populations by roofs. Values are means ± 95% C.I..

Figure 2: Effects of thermal acclimation on mitochondrial complex I / NADH dehydrogenase (A, relative decrease (%) of uncoupled respiration following rotenone addition) and complex IV / cytochrome c oxidase activity (B, respiration (pmol O_2 s^{-1} mg^{-1}) after addition of ascorbate and TMPD) in systemic hearts of cuttlefish from the English Channel and Adriatic Sea. Significant differences ($P < 0.05$) between acclimation temperatures (*N*=5-11) are marked by asterisks and between populations by roofs.

Figure 3: Temperature coefficients (Q_{10}, means ± 95% C.I.) of cardiac mitochondrial respiration from 11°C to 21°C, resolved for each substrate and for total state 3 and complex IV respiration. As there were no differences between acclimations, data were pooled for each case. Q_{10} of pyruvate and succinate are based on stimulating respiration.

Figure 4: Change of proline stimulated respiration without ADP (denoted as proton leak) and with ADP (pmol O_2 s^{-1} mg^{-1}) and relative proton leak (%, calculated as fraction of leak rates relative to coupled respiration with ADP) from 11°C to 21°C assay temperature in systemic hearts, in comparison between (A) English Channel (*N*=24) and (B) Adriatic (*N*=19-20) cuttlefish. Due to the lack of differences between acclimation treatments, data were pooled for each assay temperature and population. Values are means ± 95% C.I. Significant differences ($P < 0.05$) between assay temperatures for relative proton leak are marked by unequal letters of the same case and between populations by roofs.

Figure 5: Relative change of maximum state 3 respiration (%) following cytochrome c addition, from 11°C to 21°C assay temperature in systemic hearts, in comparison between English Channel (*N*=23-24) and Adriatic (*N*=19-20) cuttlefish. Due to the lack of differences between acclimation treatments, data were pooled for each assay temperature and population. Values are means ± 95% C.I.. Significant differences ($P < 0.05$) between assay temperatures are marked by unequal letters of the same case and between populations by roofs.

Table 1: Enzyme activities (µmol min^{-1} mg $protein^{-1}$, at 16°C) of (A) citrate synthase, (B) lactate dehydrogenase and (C) octopine dehydrogenase of cuttlefish systemic and branchial hearts, in comparison between thermal acclimations, as well as between English Channel and Adriatic Sea populations.

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Figures

Figure 1

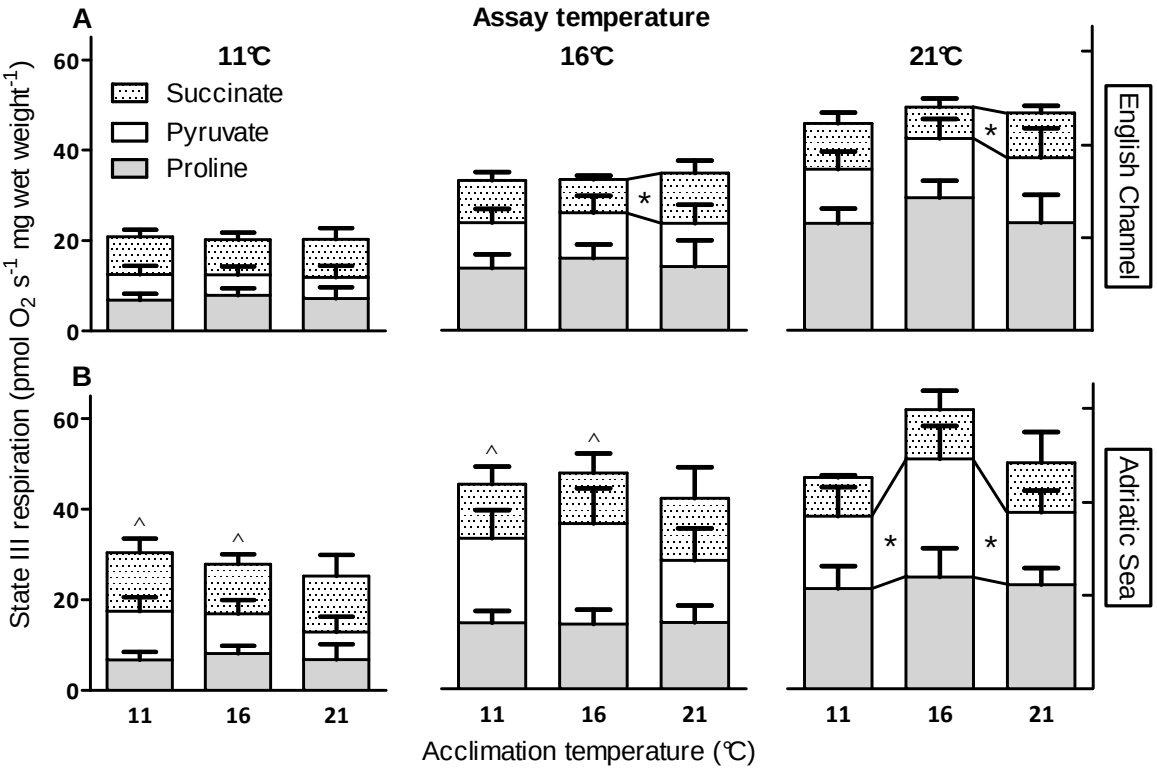

Figure 2

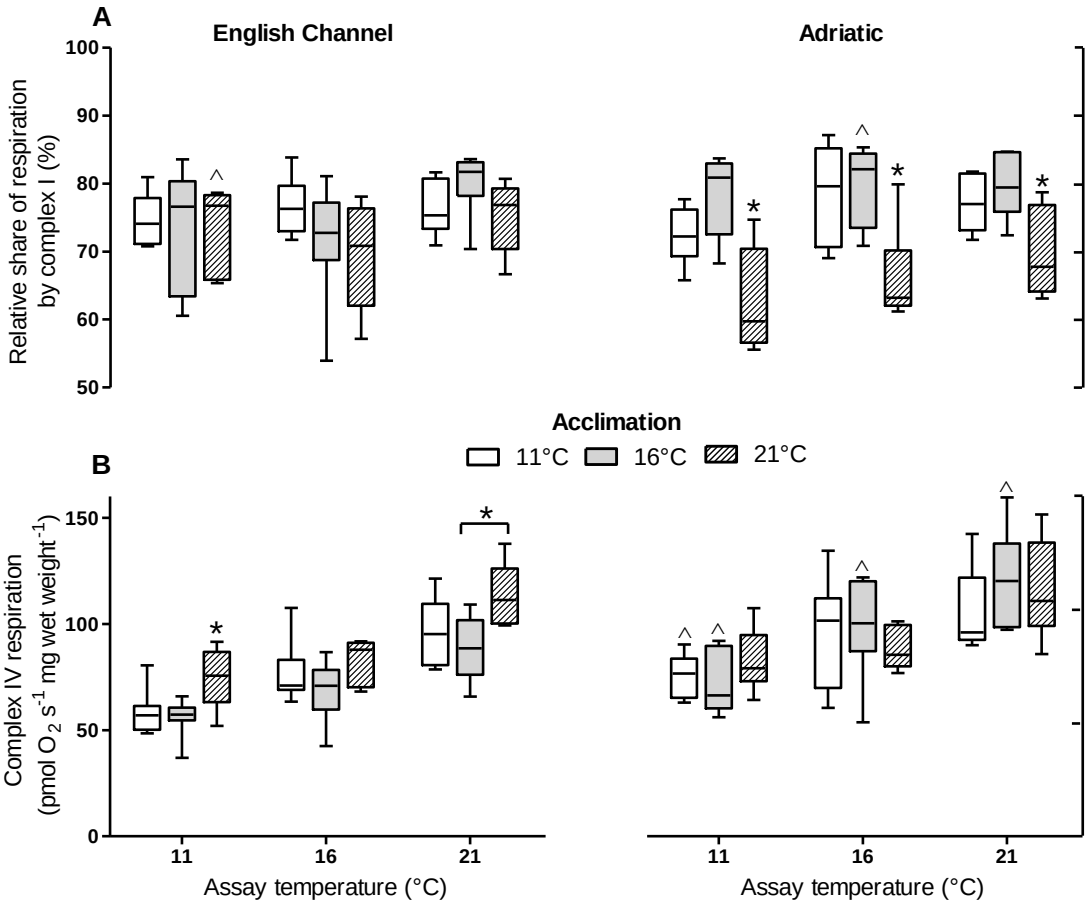

Figure 3

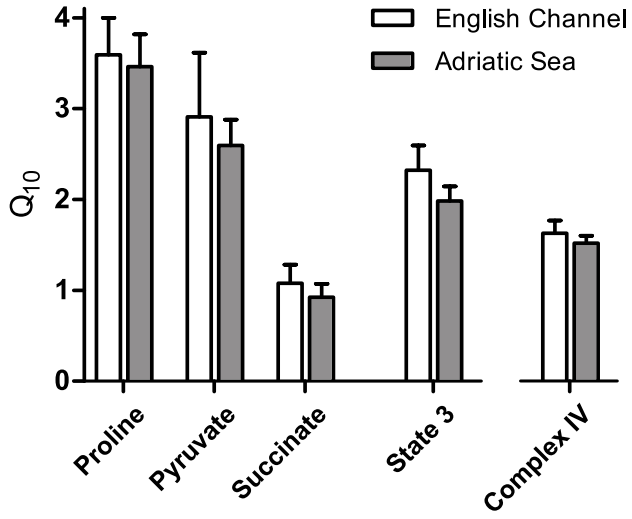

Figure 4

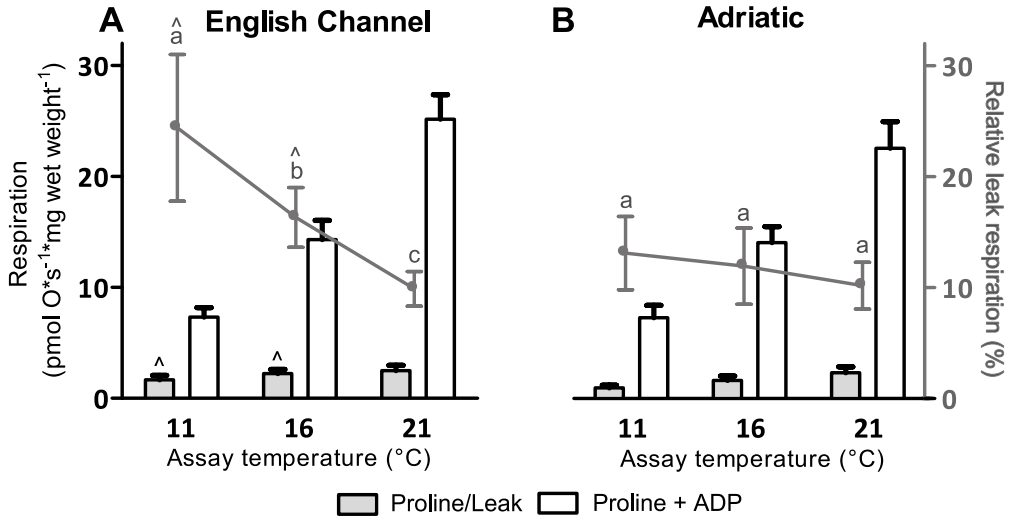

Figure 5

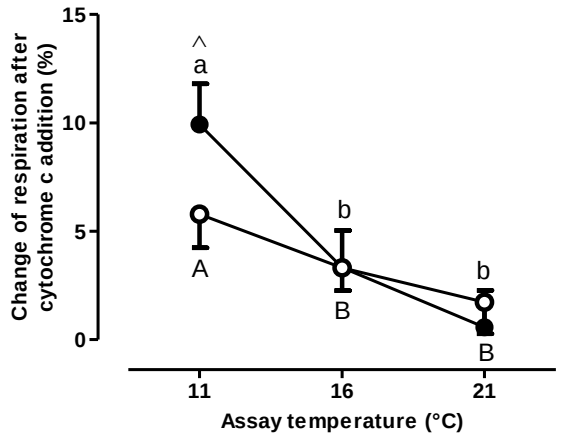

Table 1

Enzyme	Heart type	Systemic heart			Branchial heart		
		Acclimation temperature (°C)					
		11	16	21	11	16	21
Citrate synthase	English Channel	0.89 (0.82-0.96)[a]	0.73 (0.65-0.82)[b] ▲	0.74 (0.60-0.88)	0.64 (0.51-0.77)	0.78 (0.67-0.89)	0.81 (0.74-0.88)
	Adriatic Sea	1.07 (0.53-1.63)	1.01 (0.80-1.22)	0.81 (0.67-0.96)	0.63 (0.53-0.74)	0.64 (0.51-0.76)	0.78 (0.63-0.93)
Lactate dehydrogenase	English Channel	0.10 (0.07-0.14)	0.11 (0.09-0.12) [a]	0.05 (0.04-0.07)[b] ▲	0.12 (0.10-0.14) ▼	0.10 (0.09-0.12) ▼	0.08 (0.06-0.10)
	Adriatic Sea	0.13 (0.08-0.19)	0.09 (0.05-0.13)	0.08 (0.07-0.10)	0.07 (0.05-0.10)	0.07 (0.06-0.09)	0.06 (0.06-0.07)
Octopine dehydrogenase	English Channel	0.40 (0.34-0.46) ▲	0.48 (0.43-0.53)	0.39 (0.29-0.48) ▲	0.22 (0.17-0.27)	0.24 (0.20-0.27)	0.23 (0.20-0.26)
	Adriatic Sea	0.56 (0.46-0.66)	0.57 (0.35-0.80)	0.62 (0.49-0.75)	0.26 (0.18-0.33)	0.23 (0.18-0.29)	0.28 (0.23-0.34)

Values are means with 95% C.I.in parentheses. *N* = 5-17

▲/▼ indicate significant differences (*P*<0.05) between populations and unequal letters between acclimation temperatures