

RESEARCH ARTICLE

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

Michael Oellermann, Hans O. Pörtner and Felix C. Mark*

Alfred Wegener Institute for Polar and Marine Research, 27570 Bremerhaven, Germany

*Author for correspondence (Felix.Christopher.Mark@awi.de)

SUMMARY

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 crucial for the cuttlefish's competitive success in variable environments. Two genetically distinct cuttlefish populations were acclimated to 11, 16 and 21°C. 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, the thermal sensitivity of mitochondrial substrate oxidation was high for proline and pyruvate but low for succinate. Oxygen efficiency of catabolism rose as temperature changed from 11 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 and heart mass 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.

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Key words: temperature sensitivity, cephalopod, evolutionary adaptation, thermal acclimation, proton leak, lactate dehydrogenase, octopine dehydrogenase, respiration, systemic heart, branchial heart, cardiac fibres.

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INTRODUCTION

The evolution of modern cephalopods was shaped by the rising competition with marine vertebrates and led to innovations that enable high levels of performance. Concomitant increases in energy demands required extant cephalopods to optimise the 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, the design constraints led to locomotion by jet propulsion and a comparatively low blood oxygen-carrying capacity. Optimisation of performance resulted in at least some cephalopod species operating 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 because of limited

functional capacities of the circulation and ventilation system to deliver oxygen (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). Adaptive adjustment of for example ventilatory musculature and heart rate or stroke volume can compensate for temperature-induced impairment 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, required to power circulation or ventilation (Pörtner, 2002a).

Ectothermic hearts play a major role in defining the 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, the systemic hearts cover increased metabolic demands during either exercise or rising temperatures by a 2- to 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 (Schipf, 1987; Melzner et al., 2007a). Adversely, high blood viscosity, 2- to 3-fold lower blood oxygen-carrying capacities and higher resting metabolic rates compared with 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, where 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 *S. 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 lifestyle on rocky to sandy grounds down to 200 m depth. Cuttlefish are rather sluggish, grow fast (up to 2 kg), and spawn after 1 or 2 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 and 17.5°C with a genetically distinct Mediterranean population (Wolfram et al., 2006) facing a range from 10 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 *S. officinalis* heart by: (1) exploring cardiac adjustments following long-term genetic isolation in a temperate and subtropical habitat; (2) following thermal acclimation; and (3) investigating the thermal sensitivity of heart mitochondria.

MATERIALS AND METHODS

Experimental animals

Wild laid eggs of the European cuttlefish (*S. officinalis*) were collected from the temperate Oosterschelde lagoon, The Netherlands (3°56'E, 51°35'N; English Channel population) and the subtropical Venetian lagoon close to Chioggia, Adria, Italy (12°18'E, 45°13'N; Adriatic Sea population). Populations from these localities form geographically distant and genetically distinct clades without evidence of on-going genetic exchange (Wolfram et al., 2006; Pérez-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 (50–60 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 10 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±0.1°C) at suitable stocking densities (Hanley et al., 1998) under a 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 larger size, exclusively with frozen brown shrimp (*C. crangon*). Water quality parameters were monitored

weekly and kept at levels appropriate for cuttlefish culture (Hanley et al., 1998) (mean ± s.d. 10.07±0.43 mg O₂ l⁻¹, pH 8.05±0.02, salinity 32.6±0.5 psu; NH₄⁺<0.2 mg l⁻¹, NO₂⁻<0.2 mg l⁻¹, NO₃⁻<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, which were acclimated to 11, 16 or 21°C from 8 to 22 weeks. These acclimation temperatures were selected to expose European cuttlefish to their seasonal and depth-dependent temperature range, 10–17.5°C for the English Channel population and 10–25°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 material Table S2) at the end of the acclimation period (possibly due to enhanced growth rates), correlation analysis did not show clear evidence that mass differences obscured our data.

Mitochondrial respiration

Dissection and muscle fibre preparation

Animals were anaesthetised in 3% ethanol until non-responsive before culling. Subsequently, total mass, mantle length, total length and sex were recorded. The mantle cavity was opened and 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 from Kuznetsov et al., 2008) to preserve mitochondrial function. The biopsy buffer contained (in mmol l⁻¹): 2.77 CaK₂EGTA, 7.23 EGTA, 14.46 KOH, 5.77 Na₂ATP, 6.56 MgCl₂, 20 taurine, 20 imidazole, 0.5 dithiothreitol (DTT), 50 MES, 588 sucrose and 252 glycine, pH 7.4 at 26°C, 1000 mosmol l⁻¹.

Non-cardiac tissue was removed and all three hearts weighed, then approximately 50 mg of systemic heart tissue was placed into a drop of biopsy buffer on a Petri dish on ice, coarsely torn apart and dissected into small fibre bundles using two tweezers. The remaining systemic heart tissue and the branchial hearts were snap-frozen in liquid nitrogen and stored at –80°C for subsequent enzyme assays. Following a modified protocol (Saks et al., 1998), fibre bundles were immediately transferred to 1 ml biopsy buffer in a 12-well culture plate and permeabilised with 50 µg ml⁻¹ saponin (preliminary testing confirmed the appropriate saponin concentration) by gentle mixing (115 r.p.m.) 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 KH₂PO₄, 0.5 EGTA, 50 KCl, 50 NaCl, 10 MgCl₂, 20 taurine, 50 lactobionate, 350 sucrose, and 150 glycine, with 1 g l⁻¹ freshly added fatty acid-free BSA, pH 7.4 at 22°C, 1000 mosmol l⁻¹) (modified from Mommsen and Hochachka, 1981; Agnisola et al., 1991; Kuznetsov et al., 2008) and stored in ice-cold respiration medium until use.

Measurement 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 mass-specific oxygen

consumption rate ($\text{pmol O}_2\text{s}^{-1}\text{mg}^{-1}$, i.e. negative time derivative of oxygen concentration) using DatLab analysis software (Oroboros Instruments). Assays were performed consecutively at 11, 16 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 the chambers were kept between 80 $\text{nmol O}_2\text{ml}^{-1}$ (maximum stimulated respiration remained stable down to this level) and air saturation (270–340 $\text{nmol O}_2\text{ml}^{-1}$) 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}), and 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 our 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 $\mu\text{mol g}^{-1}$). 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 α -ketoglutarate (Ballantyne et al., 1981; Mommsen and Hochachka, 1981; Hochachka and Fields, 1982).

The integrity of the mitochondrial membranes was tested by the addition of cytochrome *c* (0.01 mmol l^{-1}), which would stimulate respiration in the case of damaged outer membranes. Proline-stimulated respiration without ADP (state 2) provided an estimate of mitochondrial proton leak (Ifukar et al., 2010). Uncoupling of respiration from ATP production by carbonylcyanide-*p*-(trifluoromethyl) phenylhydrazone (FCCP, up to 2.5 $\mu\text{mol l}^{-1}$) provided the maximum capacity of the electron transport chain. The loss of activity with rotenone (2.5 $\mu\text{mol l}^{-1}$) indicated NADH dehydrogenase (complex I) activity. The loss of activity with antimycin A (2.5 $\mu\text{mol l}^{-1}$) 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 material Table S1). Background auto-oxidation of the redox pair was determined for all experimental temperatures and subtracted from the final results. Suitability and concentrations of substrates were tested beforehand. All chemicals were purchased from Sigma-Aldrich (Schnellendorf, Germany).

Enzyme assays

Protein extraction and sample preparation

To extract 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%). Samples were centrifuged twice for 10 min at 6000 *g* and 4°C; supernatants were used for protein and enzyme assays; pellets 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 triplicate using 96-well flat-bottom microplates (Nunc GmbH & Co. KG, Wiesbaden, Germany), a multiplate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany) and analysing software (FLUO 32, version 4.31 R5).

Enzyme activities and protein content

Citrate synthase (EC 4.1.3.7) activity was determined (see 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), DTT was used as standard for the calculation of SH group turnover.

The activities of octopine dehydrogenase (EC 1.5.1.11) and lactate dehydrogenase (EC 1.1.1.27) indicate the capacity to form or recycle anaerobic end products in cephalopods (Grieshaber and Gäde, 1976; Storey, 1977; Storey and Storey, 1979). Octopine dehydrogenase and lactate dehydrogenase activities were determined (see Storey, 1977; 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 and 10 arginine, added for quantifying the octopine dehydrogenase reaction) followed by the addition of 20 μl 4 mmol l^{-1} pyruvate to start the reaction. Final octopine dehydrogenase activity was determined by subtracting the lactate dehydrogenase activity, which was measured simultaneously. NADH was used as a standard.

Enzyme activities were standardised to protein content (see 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). This was mixed and incubated for 10 min at room temperature (21°C); absorbance was then recorded at 595 nm. Bovine albumin serum (BSA) was used as the protein standard.

Statistical analysis

Statistical analysis was performed to spot significant differences ($P < 0.05$) using SPSS (version 14.0.1) by employing tests as follows: 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 to assess normality and homogeneity of variance, respectively. Data were expressed as means and the range of their 95% confidence interval (CI) 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:

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{\frac{10}{(T_2 - T_1)}}, \quad (1)$$

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

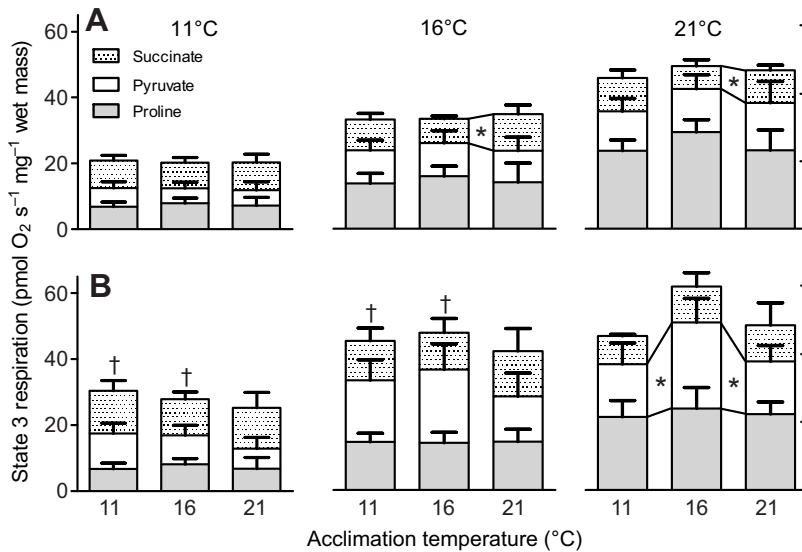


Fig. 1. Comparison of maximum state 3 respiration resolved for each substrate, after addition of ADP, at 11, 16 and 21°C assay temperature, between acclimation treatments ($N=5-11$) as well as between cuttlefish from (A) the English Channel and (B) the Adriatic Sea. Significant differences ($P<0.05$) between acclimation groups are marked by asterisks and for total state 3 respiration between populations by daggers. Values are means \pm 95% confidence interval (CI).

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 $41.1 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ (95% CI range $37.3-44.9 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$) compared with only $32.9 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($30.0-35.9 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$) in English Channel cuttlefish (ANOVA, $F_{1,128}=11.92$, $P<0.01$, calculations based on entire pooled data set). This 20% higher cardiac aerobic capacity in cuttlefish from warmer waters was most pronounced at lower assay temperatures, and was mainly due to a higher contribution of pyruvate to overall respiration (mean contribution of $15.2 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$, $13.1-17.3 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ for Adriatic cuttlefish compared with $9.0 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$, $7.8-10.2 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ for English Channel cuttlefish; Fig. 1). Consequently, the pyruvate-dependent respiration fraction constituted 35.4% (32.8–38.0%) in systemic hearts of Adriatic Sea cuttlefish but only 26.2% (24.1–28.3%) in English Channel cuttlefish. Conversely, the proline-dependent fraction was higher (45.2%, 42.5–47.8%) in English Channel than in Adriatic Sea cuttlefish (35.2%, 32.4–38.0%). The succinate-dependent fraction was similar in the two populations.

While body mass 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 material Table S2). The relative mass of systemic hearts was 0.083% (0.078–0.088%) of total mass in English Channel and 0.059% (0.056–0.062%) in Adriatic cuttlefish. Similarly, the relative mass of branchial hearts constituted 0.047% (0.044–0.050%) of total mass 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 \times heart mass) did not differ between populations (ANOVA, $F_{1,41}=0.03$, $P=0.86$) because of the larger hearts in English Channel cuttlefish.

Further, systemic hearts of Adriatic cuttlefish showed slightly higher protein-specific enzyme activities compared with English Channel cuttlefish, which was most apparent for octopine

dehydrogenase (Table 1). Enzyme activities of branchial hearts were similar between populations, with the exception of the enhanced lactate dehydrogenase activity in English Channel cuttlefish acclimated to 11 and 16°C (Table 1).

Thermal acclimation

Acclimation of cuttlefish to 11, 16 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 Adriatic Sea populations (Kruskal–Wallis, $H_2=0.94$, $P=0.63$) but caused minor shifts in substrate-dependent fractions (Fig. 1) and slight changes in cardiac enzyme activities of English Channel cuttlefish, shown by enhanced citrate synthase activity at 11°C and lower lactate dehydrogenase activity at 21°C compared with animals acclimated to 16°C (Table 1). Also, relative heart mass did not change with thermal acclimation for systemic hearts (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 and 19% lower complex I activity compared with cuttlefish acclimated to 11 and 16°C, whereas complex I activity in English Channel cuttlefish did not respond to thermal acclimation (Fig. 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 and 21°C (Fig. 2B). In Adriatic cuttlefish hearts, however, complex IV activity remained unaffected by thermal acclimation but showed between 8 and 30% higher enzyme activity at lower acclimation temperatures compared with English Channel cuttlefish (Fig. 2B).

Thermal sensitivity of cardiac mitochondria

In both cuttlefish populations, cardiac mitochondria responded similarly to acute temperature changes ranging from 11 to 21°C. State 3 respiration increased from 20.4 ($18.1-22.8$) to $46.4 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($42.9-49.9 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$) in English Channel cuttlefish and from 27.8 ($24.8-30.8$) to $51.2 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($45.3-57.1 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$) in Adriatic cuttlefish, with a mean Q_{10} of 2.3 in English Channel and 2.0 in Adriatic animals (Fig. 3). Respiration resolved for substrates revealed a high thermal

Table 1. Enzyme activities of cuttlefish systemic and branchial hearts at different acclimation temperatures and between English Channel and Adriatic Sea populations

Enzyme	Population	Systemic heart			Branchial heart		
		11°C	16°C	21°C	11°C	16°C	21°C
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)

Citrate synthase, lactate dehydrogenase and octopine dehydrogenase activities are in $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein, at 16°C.

Values are means with 95% confidence intervals in parentheses; $N=5-17$.

^{*}Significant differences between English Channel and Adriatic Sea populations ($P<0.05$). Different lowercase letters indicate significant differences between acclimation temperatures ($P<0.05$).

sensitivity of proline- and pyruvate-stimulated respiration but a low thermal sensitivity of succinate-stimulated respiration (Fig. 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 (Fig. 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 by ascorbate and TMPD) showed low thermal sensitivity (Fig. 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 (Fig. 4). Outer membrane integrity, tested by cytochrome *c* addition, decreased with increasing assay temperature in both

cuttlefish populations (Fig. 5). Further, English Channel cuttlefish showed more defective outer membranes and higher relative proton leak at 11°C assay temperature compared with Adriatic cuttlefish (Fig. 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 had lower aerobic and slightly lower anaerobic capacities (Fig. 1; Table 1) that may reduce their cardiac output and hence lower their ability to increase heart rate 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 capacity by having larger hearts. Compensatory increases of heart mass are also common in teleost fish living at cooler temperatures (Foster et al.,

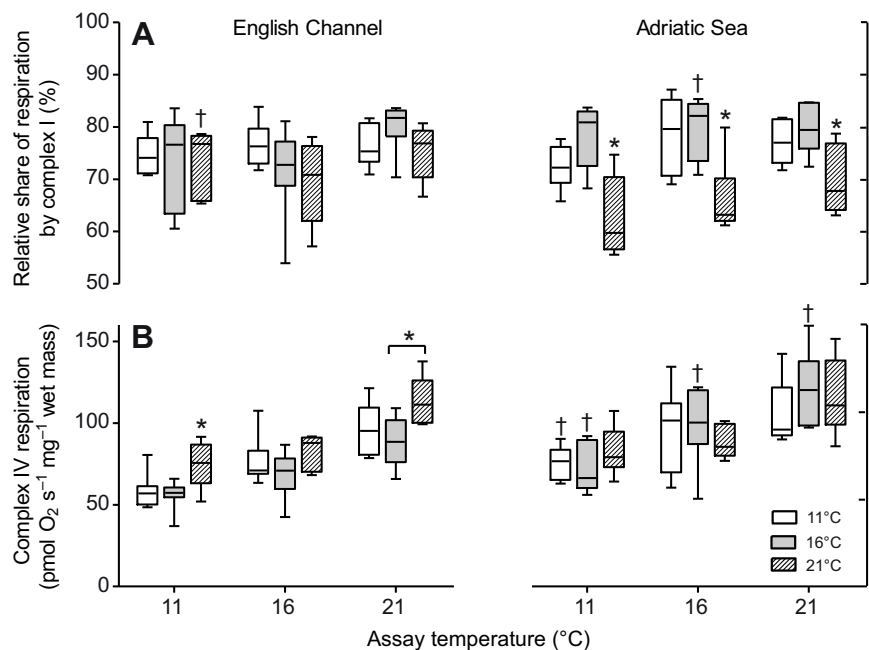


Fig. 2. Effects of thermal acclimation (11, 16 and 21°C) on mitochondrial complex I/NADH dehydrogenase activity (A, relative decrease of uncoupled respiration following rotenone addition) and complex IV/cytochrome *c* oxidase activity (B, respiration after addition of ascorbate and TMPD) in systemic 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 daggers.

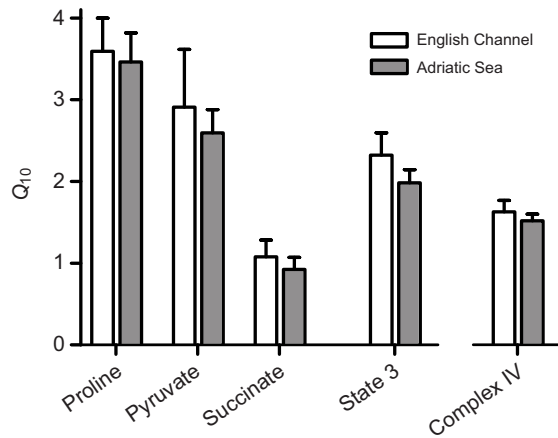


Fig. 3. Temperature coefficients (Q_{10} , means \pm 95% CI) of cardiac mitochondrial respiration from 11 to 21°C, resolved for each substrate and for total state 3 and complex IV respiration. As there were no differences between acclimations, data were pooled for each case. Q_{10} of pyruvate and succinate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

1993; Driedzic et al., 1996). Larger hearts support the pumping of larger blood volumes per stroke; thus, they can compensate for low energetic capacities and also for rising blood viscosities at colder temperatures (Goolish, 1987; Driedzic et al., 1996). Therefore, cuttlefish heart function in the cold may be sustained through an increase in organ size, rather than through an increase of cellular energetic capacity.

Furthermore, the finding of a predominant oxidation of proline in systemic hearts of temperate cuttlefish but enhanced pyruvate oxidation in subtropical cuttlefish concurs well with findings for cold-adapted fish showing 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 by the heart and threatens 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, one mole of proline requires half a mole of dioxygen to oxidise it to glutamate before entering the Krebs cycle, thus decreasing its oxygen efficiency (Mommensen and Hochachka, 1981). Therefore, impaired oxygen supply at higher temperatures or environmental hypoxia, which cuttlefish often face in northern

Adriatic lagoons (Diaz, 2001; Sorokin and Dalocchio, 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 capacity (i.e. state 3 respiration, Fig. 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. However, in parallel with cardiac capacity, routine metabolic rate declined by 2–3.5 times from 20 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 temperature and thus match a reduction of energy turnover during the cold season, marked by a reduced growth rate of cuttlefish during winter (Le Goff and Daguzan, 1991).

Mitochondrial complexes

The 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). Because of 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 frequently face critically low intracellular oxygen concentrations, particularly when high temperatures induce tissue hypoxia (Pörtner, 2001). Gnaiger and colleagues argued that an excess capacity of complex IV sustains the high affinity for oxygen in mitochondria (Gnaiger et al., 1998). Therefore, the increase of complex IV capacity in systemic hearts of English Channel cuttlefish following warm acclimation to 21°C (Fig. 2B) probably enhanced oxygen affinity, thus supporting oxygen diffusion to mitochondria at high and hypoxia-inducing temperatures. This response is in agreement with the finding that

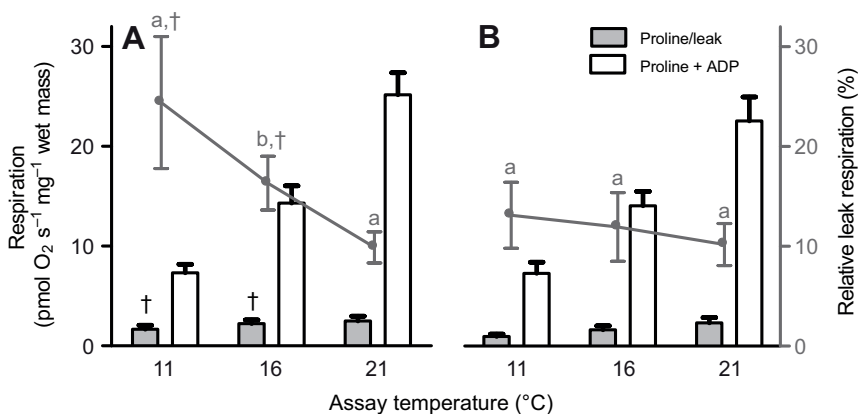


Fig. 4. Change of proline-stimulated respiration without ADP (denoted as proton leak) and with ADP, and relative proton leak (calculated as the fraction of leak rate relative to coupled respiration with ADP) from 11 to 21°C assay temperature in systemic hearts, for (A) English Channel ($N=24$) and (B) Adriatic ($N=19-20$) cuttlefish. Because of the lack of differences between acclimation treatments, data were pooled for each assay temperature and population. Values are means \pm 95% CI. Significant differences ($P < 0.05$) between assay temperatures for relative proton leak are marked by different lowercase letters and between populations by daggers.

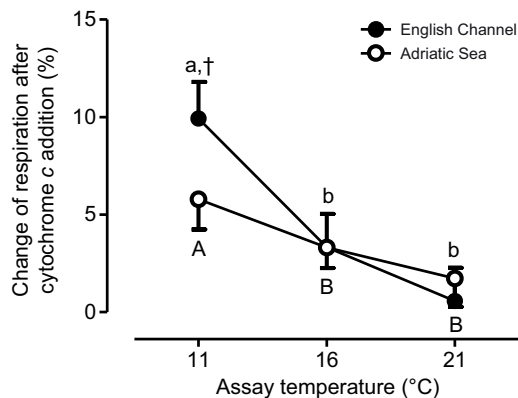


Fig. 5. Relative change of maximum state 3 respiration (%) following cytochrome *c* addition, from 11 to 21°C assay temperature in systemic hearts, for English Channel ($N=23-24$) and Adriatic ($N=19-20$) cuttlefish. Because of the lack of differences between acclimation treatments, data were pooled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

some fishes are capable of increasing cardiac complex IV activity following warm acclimation, as seen in the highly hypoxia-tolerant carp (Cai and Adelman, 1990) and 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, suppression of complex I capacity following warm acclimation in Adriatic cuttlefish (Fig. 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 to reduce temperature-induced hypoxia or harmful oxygen stress in cuttlefish cardiac tissues.

Thermal sensitivity of cardiac mitochondria

Substrate oxidation

In the summer, cuttlefish pass a steep thermocline during their daily vertical migration, with possible temperature changes of up to 2°C m^{-1} (e.g. Adriatic Sea: 23 to 14°C down to 80m; English Channel: 19 to 11°C down to 40m) (Artegiani et al., 1997; Sharples et al., 2001). Our findings demonstrate that noticeable increases of mitochondrial respiration (state 3) from 11 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 (Fig. 3). The substrate-dependent effect on the thermal sensitivity of oxidative pathways may allow mitochondria to produce aerobic energy over a broader range of temperatures. While pathways that are less temperature sensitive (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 decline at low temperatures, the *in situ* supply of succinate may be sustained by amino acids such as glutamate, ornithine or arginine that are well oxidised 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 from carbohydrate oxidation, which comes into effect in the warmth (Sephton et al., 1990; Sephton and Driedzic, 1991).

The predominance of pyruvate and proline oxidation at higher temperatures (Fig. 1) improves oxygen-efficient production of ATP and probably reduces the threat of tissue hypoxia. After entering mitochondria, proline is oxidised to glutamate and then enters the Krebs cycle *via* α -ketoglutarate, yielding 4–5 NADH and 1 FADH₂ (i.e. assuming no diffusive loss of products), similar to pyruvate, which yields 4 NADH and 1 FADH₂ 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 FADH₂ and, thus, diverts only 50% of its electrons to complex I when fully catabolised to oxaloacetate. Oxygen-efficient ATP production may be advantageous at high temperatures when oxygen becomes limiting (for a review, see Pörtner, 2010) and is 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 (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) (Figs 4, 5). In contrast to the present observations, relative proton leak increases with temperature in mitochondria isolated from hearts, red muscle and liver of fish (Hardewig et al., 1999; Fanguie et al., 2009; Hilton et al., 2010), gills of Antarctic bivalves (Pörtner et al., 1999) or the body wall of lugworms (Sommer and Pörtner, 2002). Our finding therefore 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* (Fig. 5), which crosses the outer membrane by diffusion only (Gellerich et al., 2000). Such evident changes of the outer membrane permeability probably affected the inner mitochondrial membrane too and hence supported the observed decline of relative proton leak with rising temperature. 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 (Figs 4, 5). Their

mitochondria therefore operate less efficiently and in a more 'costly' way at cool temperatures. Conversely, a higher 'futile cycling' through proton leak would make them more responsive to sudden increases in workload. This may be needed less in warm-acclimated hearts.

CONCLUSION

Our study on cuttlefish heart fibres and their mitochondria revealed the 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 hypoxaemic 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. The lack of cold compensation by adjusting mitochondrial or enzyme capacities during cold acclimation, however, suggests decreased cardiac energetic capacity during the cold season. Overall, we conclude that the observed flexibility of cardiac function, based on specific cephalopod-type characteristics, ensures cardiac power output and systemic oxygen delivery at various temperatures. It thus conforms to the capacity of *S. officinalis* to tolerate a broad range of temperatures and supports their ability to contend with fishes – their prime competitors – in rapidly changing environments.

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Table S1. Action and concentrations of the agents used to assay mitochondrial respiration of permeabilised heart fibres from the European cuttlefish

Agent	Action	Final concentration
Substrates		
Proline	Amino acid substrate	5 mmol l ⁻¹
Pyruvate	Carbohydrate substrate	5 mmol l ⁻¹
Succinate	Carbohydrate substrate	10 mmol l ⁻¹
ADP	Substrate for ATP generation	2.5 mmol l ⁻¹
Cytochrome <i>c</i>	Electron carrier, tests for outer membrane integrity	0.01 mmol l ⁻¹
TMPD	Complex IV substrate	5 mmol l ⁻¹
Ascorbate	Complex IV substrate	20 mmol l ⁻¹
Inhibitors		
Antimycin	Inhibits complex III, to test non-mitochondrial respiration	2.5 μmol l ⁻¹
Rotenone	Complex I inhibitor	2.5 μmol l ⁻¹
Oligomycin	Inhibits ATP synthase, to test proton leak	2 μg ml ⁻¹
Uncoupler		
FCCP	Uncouples the electron transport system electron flow from ATP generation by relieving the proton gradient	2.5 μmol l ⁻¹

Table S2. Measurements of the European cuttlefish *Sepia officinalis* used in acclimation experiments

Identifier	Origin	Acclimation temperature (°C)	Sex	Date of sampling	Total mass (g)	Mantle length (cm)	Systemic heart mass (mg)	Branchial heart #1 mass (mg)	Branchial heart #2 mass (mg)	Comments
A06	Adriatic	11	Male	7/07/2009	90.22	8.8	58.3	38.5	33.5	
A10	Adriatic	11	Male	14/07/2009	63.34	8.0	39.3	25.4	28.9	
A13	Adriatic	11	Male	16/07/2009	83.75	8.5	49.3	37.0	34.3	Animal was inking during capture
A30	Adriatic	11	Female	21/09/2009	99.20	9.1	58.6	38.0	37.4	Animal was inking during capture
A63	Adriatic	11	Female	14/07/2010	110.53	9.3	58.3	39.7	39.2	Animal was inking during capture
A64	Adriatic	11	Male	19/07/2010	78.47	8.2	40.1	27.8	30.9	Animal was inking during capture
A65	Adriatic	11	Female	20/07/2010	86.40	8.0	43.1	34.9	35.3	Animal was slightly inking during capture
A69*	Adriatic	11	Male	22/11/2010	48.20	–	24.6	19.9	18.4	
A07	Adriatic	16	Female	8/07/2009	46.90	6.8	30.5	23.7	22.4	
A09	Adriatic	16	Male	13/07/2009	40.57	7.0	27.0	11.6	19.5	Animal was inking during capture
A14	Adriatic	16	Male	20/07/2009	72.52	8.2	44.9	24.7	27.5	Animal was inking during capture
A19	Adriatic	16	Female	30/07/2009	77.88	7.9	46.4	42.2	38.5	Animal was inking during capture, very well developed gonads
A24	Adriatic	16	Female	10/08/2009	59.11	7.4	37.1	41.6	42.6	
A46*	Adriatic	16	Female	20/04/2010	50.10	7.1	27.3	18.3	18.7	Animal was inking during capture
A47*	Adriatic	16	Male	21/04/2010	49.87	7.0	27.1	15.0	16.5	Animal was inking during capture
A48*	Adriatic	16	Male	26/04/2010	59.67	7.5	33.7	24.5	21.8	Animal was inking during capture
A49*	Adriatic	16	Female	27/04/2010	62.66	7.2	33.0	20.9	24.7	Animal was inking during capture
A50*	Adriatic	16	Male	28/04/2010	52.05	6.9	29.1	22.1	24.3	Animal was inking during capture
A51*	Adriatic	16	Female	29/04/2010	41.34	6.2	25.6	13.6	16.7	Animal was inking during capture
A61	Adriatic	16	Male	12/07/2010	109.93	9.5	77.4	42.1	44.5	Animal was inking during capture
A62	Adriatic	16	Male	13/07/2010	144.00	10.5	101.1	56.3	56.2	
A52*	Adriatic	21	Female	26/05/2010	148.82	10.5	74.7	40.3	43.8	
A53*	Adriatic	21	Male	31/05/2010	128.71	10.0	68.7	40.0	39.8	
A54*	Adriatic	21	Female	2/06/2010	228.17	11.2	113.2	65.3	78.8	Female had proper developed gonads with yellowish transparent eggs
A55*	Adriatic	21	Male	3/06/2010	252.92	12.7	135.6	74.7	74.3	
A56*	Adriatic	21	Male	7/06/2010	149.30	10.4	75.7	55.3	47.6	
A57*	Adriatic	21	Male	9/06/2010	150.37	10.1	75.6	41.5	45.3	
A58	Adriatic	21	Female	7/07/2010	147.75	10.0	86.7	58.7	56.9	Female had eggs
A59	Adriatic	21	Female	8/07/2010	226.54	11.3	140.7	82.9	79.9	Animal was inking during capture, female had eggs
A60	Adriatic	21	Male	9/07/2010	198.63	11.6	109.0	69.4	74.8	Animal was inking during capture
A66	Adriatic	21	Female	21/07/2010	184.21	10.8	145.4	87.7	87.2	Female already mated as there were sucker marks on the head, had hardly ink in ink sac
A67	Adriatic	21	Female	23/07/2010	144.90	9.5	108.5	57.4	59.1	Female already mated as there were sucker marks on the head, had hardly ink in ink sac
A68	Adriatic	21	Female	26/07/2010	248.32	12.2	161.3	96.0	101.3	
A08	Oosterschelde	11	Male	10/07/2009	59.09	7.3	42.8	23.9	27.5	
A11	Oosterschelde	11	Male	15/07/2009	79.46	7.9	63.5	41.2	38.3	
A12	Oosterschelde	11	Female	21/07/2009	89.19	8.3	63.2	37.5	39.1	
A23	Oosterschelde	11	Male	6/08/2009	96.64	8.9	81.2	51.3	48.5	Animal was inking during capture
A25	Oosterschelde	11	Male	12/08/2009	112.12	9.5	70.1	47.0	42.0	
A26	Oosterschelde	11	Male	13/08/2009	77.84	8.5	60.1	37.0	38.5	
A27	Oosterschelde	11	Male	18/08/2009	92.99	8.6	73.0	43.4	45.8	Animal was inking during capture
A28	Oosterschelde	11	Male	8/09/2009	79.98	8.1	65.1	38.1	36.0	
A29	Oosterschelde	11	Male	17/09/2009	83.31	8.7	83.6	53.9	50.6	
A04	Oosterschelde	16	Female	4/06/2009	72.46	8.5	–	–	–	

A05	Oosterschelde	16	Female	17/06/2009	84.50	8.6	57.3	31.6	31.4	Animal was inking during capture
A15	Oosterschelde	16	Female	22/07/2009	97.70	8.9	61.6	38.4		
A18	Oosterschelde	16	Male	29/07/2009	108.29	9.1	111.4	46.4	43.8	Animal was inking during capture
A31	Oosterschelde	16	Male	22/09/2009	125.10	9.3	114.5	50.4	59.3	
A32	Oosterschelde	16	Male	23/09/2009	79.75	7.7	73.1	34.4	33.8	
A33	Oosterschelde	16	Female	24/09/2009	253.83	12.5	315.7	130.0	134.3	Female had proper developed gonads with yellowish transparent and white eggs
A34*	Oosterschelde	16	Female	28/08/2009	164.90	9.3	133.6	93.5	86.7	
A35	Oosterschelde	16	Male	29/09/2009	155.89	10.2	131.5	65.4	68.0	Animal was inking during capture
A36	Oosterschelde	16	Female	30/09/2009	128.38	9.1	97.3	54.7	56.1	Animal was inking during capture
A37	Oosterschelde	16	Female	1/10/2009	165.94	10.3	183.0	90.1	87.0	
A38	Oosterschelde	16	Male	5/10/2009	164.39	11.4	167.9	79.0	70.0	
A39*	Oosterschelde	16	Male	6/10/2009	278.93	12.6	214.8	127.8	133.0	
A40*	Oosterschelde	16	Male	7/10/2009	177.30	11.0	142.2	87.4	80.5	
A41*	Oosterschelde	16	Male	8/10/2009	178.59	10.5	123.9	80.8	89.8	Animal was slightly inking during capture
A42*	Oosterschelde	16	Female	12/10/2009	225.04	10.9	183.3	108.6	116.9	
A43*	Oosterschelde	16	Male	13/10/2009	258.39	12.3	210.0	131.8	138.1	Animal was slightly inking during capture
A44*	Oosterschelde	16	Female	14/10/2009	299.15	12.8	194.7	161.2	133.2	Female had proper developed gonads with yellowish transparent eggs
A45*	Oosterschelde	16	Female	15/10/2009	276.24	12.3	184.6	146.7	155.6	Female had proper developed gonads with yellowish transparent eggs
A16	Oosterschelde	21	Female	27/07/2009	71.33	7.9	56.8	36.2	32.7	
A17	Oosterschelde	21	Male	28/07/2009	83.59	8.1	77.7	41.4	39.5	Heart muscle was relatively tough
A20	Oosterschelde	21	Female	3/08/2009	102.13	8.5	75.5	45.7	44.5	
A21	Oosterschelde	21	Male	4/08/2009	124.66	9.9	106.3	62.3	67.9	
A22	Oosterschelde	21	Male	5/08/2009	123.77	8.9	110.7	65.3	60.4	

Individuals marked with asterisks were used for morphometric statistics but not for measurement of mitochondrial respiration.