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EFFECTS OF TEMPERATURE ON MITOCHONDRIA FROM ABALONE (GENUS *HALIOTIS*): ADAPTIVE PLASTICITY AND ITS LIMITS

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Summary

The effects of temperature on mitochondrial oxygen consumption, membrane fluidity and cytochrome *c* oxidase activity were measured for five species of eastern Pacific abalone (genus *Haliotis*) found at different latitudes and tidal heights. Mitochondria were isolated from freshly collected individuals and from specimens that had been acclimated in the laboratory to temperatures spanning the extremes of each species' known habitat temperature range. The temperatures at which Arrhenius plots of respiration rate of mitochondria from freshly collected abalone exhibited sharp breaks in slope were found to correlate with the habitat temperature at the time of capture of each species. Membranes isolated from freshly collected abalone living at warm temperatures (*Haliotis cracherodii* and *H. corregata*) were significantly less fluid (as determined by the fluorescence polarization of the probe 1,6-diphenyl 1,3,5-hexatriene) than were membranes from species captured at cooler temperatures (*H. rufesens* and *H. kamtschatkana kamtschatkana*). Laboratory acclimation significantly shifted the temperature of mitochondrial thermal inactivation in an adaptive manner in the eurythermal species, *H. fulgens*, *H. corregata* and *H. rufesens*, but did not alter this property significantly for mitochondria from the stenothermal species, *H. k. kamtschatkana*. Laboratory acclimation resulted in temperature-compensatory changes in membrane fluidity in all species except *H. rufesens*. The temperatures at which cytochrome *c* oxidase activity was inactivated also shifted in an adaptive manner in some species. Acclimation of mitochondrial respiration, membrane fluidity and cytochrome *c* oxidase activity occurred only over the ranges of temperature at which each species is common, suggesting that there is a relationship between acclimatory ability and the biogeographical distribution of congeneric species.

Introduction

Many marine organisms encounter seasonal and shorter-term fluctuations in environmental temperature (Prosser and Heath, 1991; Cossins and Bowler, 1987). For ectotherms, the maintenance of physiological function over a range of body temperatures requires seasonal or even daily adjustments in both the rates of physiological processes and the biochemical composition of the organism (Hochachka and Somero, 1984; Hazel

Key words: temperature, acclimation, mitochondria, abalone, *Haliotis* sp.

and Williams, 1990). However, acclimatory ability has its limits in all species. Therefore, although living organisms have been found in all habitats examined where water is in its liquid state, from geothermal hot springs to the Antarctic, no one species has been discovered that is able to remain metabolically active over the entire range of liquid water temperatures (Cossins and Bowler, 1987; Prosser and Heath, 1991).

It is therefore clear that acclimatory capacity has a large genetic component. However, the genetically determined limits of temperature-acclimatory capacity have not been thoroughly examined. With this study, we examine several mechanisms of thermal acclimation in one group of closely related marine invertebrates, abalone, to explore the relationship between acclimatory capacity (and its limits) and habitat temperature.

Abalone (Haliotidae) are ideal organisms for exploring relationships between habitat temperature and acclimatory ability. They live in habitats not only with different absolute temperatures, but also with differences in temperature range (Table 1). That is, some species are more eurythermal than others. For example, although three species of eastern Pacific abalone [*Haliotis fulgens* (green), *H. cracherodii* (black) and *H. corregata* (pink)] are found in warm temperate habitats, green and black abalone experience a wider range of temperatures than do pink abalone because green and black abalone occur in the intertidal zone, whereas pink abalone are restricted to the subtidal zone (Leighton, 1974; Cox, 1962; Owen *et al.* 1971). Similarly, both *H. rufesens* (red) and *H. k. kamtschatkana* (pinto) are found in cool temperate habitats. However, red abalone are occasionally exposed to elevated temperatures (18˚C) in the southern part of their distribution, whereas pinto abalone, which are not found south of Point Conception and are rare south of Oregon, are only infrequently exposed to temperatures above 12˚C. Furthermore, pinto abalone are found intertidally only in the Gulf of Alaska and along the northern coast of British Columbia and are probably quite stenothermal (Paul and Paul, 1980). On the basis of these distributions, the order of eurythermality of the abalone studied here is approximately: green, black > red, pink > pinto.

In this study, we examined the effects of temperature on mitochondrial oxygen consumption, the fluidity of mitochondrial membranes and the activity of cytochrome *c*

Table 1. *Habitat and treatment temperatures used to acclimate four species of abalone*

	Habitat temperature (°C)		Acclimation temperature (°C)		
Species (common name)	Biogeographical range	Range where most abundant	Control	Maximal	Minimal
H. fulgens (green)	14–27	18–23	20	26	14
H. corregata (pink)	12–23	12–20	20	23	11
H. cracherodii (black)	12–25	18–22	–	–	–
H. rufesens (red)	8–18	10–16	12	20	5
H. k. kamtschatkana (pinto)	4–14	6–10	10	14	5

All temperatures were controlled within 1°C of reported values.

Habitat temperature data from Ricketts *et al.* (1985), Leighton (1974; green, pink and red abalone), Hines *et al.* (1980; black abalone) and Paul and Paul (1980; pinto abalone).

oxidase in mitochondria isolated from abalone that were either freshly collected or acclimated to their maximal, minimal or capture-habitat temperatures. Experimental temperatures were selected to allow a comparison between the maximal temperature range a species experiences and the range of temperatures at which each species is common (Table 1).

Previous studies of the biogeographical distributions of marine organisms have revealed that rare individuals may be found outside the range where most of their conspecifics occur (Vernberg, 1962). Reported biogeographical distributions of marine organisms, especially species that are separated along latitudinal (and therefore thermal) gradients, probably overestimate the habitat temperature range experienced by the majority of individuals of a given species. One way to address this issue is to define the habitat temperature range of a species both by its full biogeographical range and by the range of temperatures where the species is most common.

Mitochondria are an ideal model system for comparing potentially acclimatable processes. Previous studies have shown that mitochondrial oxygen consumption is an excellent indicator of both temperature adaptation and thermal acclimation (Wodtke, 1976; van den Thillart and Modderkolk, 1978; Dahlhoff *et al.* 1991). For example, mitochondrial oxygen consumption from deep-sea hydrothermal vent invertebrates acclimatized to elevated temperatures is less affected by high temperatures than is mitochondrial respiration of species acclimatized to typical deep-sea temperatures (2–4°C; Dahlhoff *et al.* 1991). The effects of temperature on the activities of respiratory proteins of ectotherms have also been positively correlated with habitat and acclimation temperature (Hazel, 1972; Geiser and McMurchie, 1985; Wodtke, 1976; Dahlhoff *et al.* 1991).

The fluidity and composition of mitochondrial membranes are known to be altered in response to habitat and acclimatory temperature changes (Cossins *et al.* 1980; Cossins, 1983; Hazel and Williams, 1990). All lipid bilayers are extremely sensitive to temperature changes, and temperature-induced perturbations in membrane structure can seriously disrupt physiological function (Hazel, 1988; Hazel and Williams, 1990; Williams and Hazel, 1992). Previous studies of biological membranes from organisms with varying body temperatures have shown that membrane fluidity exhibits a high degree of compensation to temperature, a phenomenon defined as homeoviscous adaptation (Sinensky, 1974; Hazel, 1988; Cossins and Bowler, 1987).

In these and other studies, measurements of membrane fluidity have been made using physical methods such as fluorescence polarization. In our study, we also use this method to determine the fluidity of mitochondrial membranes isolated from abalone acclimatized and acclimated in different ways. In this method, the movement within the bilayer of a probe, such as 1,6-diphenyl 1,3,5-hexatriene (DPH), incorporated into the membrane is determined as a function of temperature. The strength of the polarization signal is related to membrane fluidity: the higher the membrane fluidity, the lower is the emission of polarized light from DPH molecules within the membrane (Shinitzky and Barenholtz, 1978).

Previous studies of adaptive alterations in mitochondrial function (respiration, activities of respiratory proteins) and structure (membrane fluidity) in response to habitat-

or acclimatory-induced differences in body temperature have focused either on intraspecific comparisons or on comparisons of membranes of phylogenetically diverse organisms (reviewed in Hazel, 1988; Hazel and Williams, 1990). No studies have yet examined the effects of temperature on the structure and function of mitochondria from closely related species living in different thermal habitats and/or acclimated to different temperatures. The examination of mitochondria from closely related species experiencing relatively small differences in body temperature may reveal whether differences in habitat temperature of only a few degrees Celsius induce adaptive changes in mitochondrial function and structure and whether eurythermal and stenothermal species differ in the range of temperatures over which acclimation occurs.

Materials and methods

Animal collection and maintenance

Abalone were obtained using snorkel or SCUBA, except for *Haliotis fulgens* specimens, which were obtained from Dr David Leighton at San Diego, CA, USA. An effort was made to capture individuals at a site that represented their peak abundance. The temperatures of capture are reported in Table 2. *H. corregata* were collected at Point Loma, CA. *H. cracherodii* were collected at Diablo Canyon, CA. *H. rufesens* were collected at Point Mendicino, CA, and *H. k. kamtschatkana* were collected near Friday Harbor, WA. All specimens were obtained between 15 July and 1 September 1991. Abalone were maintained in controlled-temperature, recirculating aquaria (Aqualogic, San Diego, CA) at Oregon State University at their capture temperature (±1˚C) and were killed within 7 days of capture. Acclimated individuals were maintained at their experimental temperature (±1˚C) in aquaria located at Oregon State University's Hatfield Marine Science Center. All species were fed *ad libitum* on an identical diet of local kelps, including *Egregia menzisii*, *Macrocystis integrifolia* and *Gigartina* sp. Light levels were controlled as is natural for the latitude of Oregon State University (approximately 45˚N), although acclimation series were conducted at random to minimize photoperiod effects. At least five individuals of each species were acclimated to either their maximal or minimal habitat temperature. These temperatures are shown in Table 1. Abalone were also maintained at their capture temperature, except for *H. fulgens* individuals, which

Table 2. *The Arrhenius break temperature of succinate-supported oxygen consumption of mitochondria from abalone captured at different temperatures*

Species	Capture temperature (°C)	Arrhenius break temperature (°C)	
		Coupled rates	Uncoupled rates
Pink	20	38.9±0.6	36.5±3.1
Black	18	36.9±3.1	38.8±2.6
Red	12	31.6±3.0	30.7±3.4
Pinto	9	30.5±4.1	30.2±2.9

Data shown are means ± S.E., *N*=5 individuals for all species.

were not collected from the field and were instead held at their culture temperature (20˚C). All individuals, both experimental and control, were held at these temperatures for 42±4 days before being killed. *H. cracherodii* were not acclimated because of a limited supply of this species.

Isolation of mitochondria

Mitochondria were isolated from the hepatopancreas by differential centrifugation in a medium consisting of 0.4mol l^{-1} sucrose, 0.4mol l^{-1} mannitol, 0.5mmol l^{-1} EGTA, 10mmol l^{-1} Hepes, pH7.4 at 20˚C, and 1% (w/v) bovine serum albumin (BSA). Animals were chilled on ice for 20min before removal from their shells. Hepatopancreas tissue was removed and immediately homogenized in 5 vols of ice-cold isolation medium using a Potter–Elvehjem homogenizer (Wheaton Scientific, Millville, NJ) driven by a drill press. The homogenate was centrifuged (9000 ***g*** for 10min) to remove (soluble) digestive enzymes as quickly as possible. This initial pellet was resuspended in isolation medium and centrifuged to remove large cellular debris (900 ***g*** for 10min). Mitochondria were subsequently pelleted from the resulting supernatant by centrifugation for 10min at 10000 ***g***. This pellet was resuspended in buffer and washed in an identical high-speed spin. The final pellet was resuspended in a minimal volume of buffer and used immediately for respiration measurements. A portion of these mitochondria was frozen at −80˚C for use in total protein determinations, cytochrome *c* oxidase assays and membrane fluidity measurements.

Respiration measurements

Oxygen consumption measurements were made with a Clark-type O_2 electrode (Strathkelvin Instruments 1302 microcathode electrode) in a glass water-jacketed chamber. Mitochondrial respiration was assayed in a medium of 0.5mol l^{-1} glycine, 0.15mol l^{-1} KCl, 5mmol l^{-1} $MgCl_2$, 10mmol l^{-1} potassium phosphate and 50mmol l^{-1} Hepes (pH7.2 at 20˚C). The rationale for the use of this medium is given in O'Brien and Vetter (1990). Respiration was measured at 5˚C increments within the range 5–55˚C. Duplicate determinations were made at each temperature for each individual. At each temperature, the reaction was started by the addition of 10–20 μl of fresh mitochondria to temperature-equilibrated assay medium (final volume 1.2ml). 1min after addition of mitochondria, 4mmol l^{-1} succinate was added to stimulate respiration. 1min after the addition of succinate, the respiratory uncoupler carbonylcyanide-*p*-(trifluoromethyl)phenylhydrazone (FCCP) was added to determine 'uncoupled' (State 3) rates.

Oxygen electrode calibration

The relationship between the amount of dissolved oxygen present in air-saturated media and the assay temperature was determined by the method of Dahlhoff *et al.* (1991). A standard curve was generated by measuring the oxygen concentration of mitochondrial assay medium at 5˚C increments from 10 to 40˚C. At least two oxygen determinations were made for each 5˚C increment, and the electrode was zeroed at each temperature.

The resulting standard curve for the mitochondrial assay medium was approximated by a line with the equation:

$$y = 310 - 3.87x \ , \quad (1)$$

where y is the number of atoms of oxygen per ml, and x is temperature in ˚C. Equation 1 was used to calculate the oxygen content of the medium at each assay temperature.

Protein concentrations

Total protein concentrations of mitochondrial preparations were determined by the bicinchoninic acid (BCA) method (Pierce Co., Rockford, IL). A correction was made to account for the presence of BSA in the isolation medium.

Fluidity measurements

Steady-state fluorescence polarization of the hydrophobic fluorophore 1,6-diphenyl 1,3,5-hexatriene (DPH) was measured by the method of Shinitzsky and Barenholtz (1978) using a Perkin–Elmer LS 50 luminescence spectrophotometer in Dr Jeffrey Hazel's laboratory at Arizona State University. This spectrophotometer was equipped with a water-jacketed cuvette holder, and measurement temperature was maintained to within 1˚C using a digital thermocouple mounted on the lid of the cuvette. Excitation of DPH was at 364nm, and emission was measured at 430nm (10nm slit width). Fluorescence polarization values were calculated using the software provided by Perkin–Elmer for this spectrophotometer. This software corrects for both light scattering and differences in excitation and emission monochrometers. The data reported in this paper are therefore appropriately corrected polarization values. Specific details of these corrections are discussed in O'Brien *et al.* (1991).

Mitochondrial preparations, stored frozen at −80˚C, were diluted in 10mmol l^{-1} Hepes (pH7.4 at 20˚C) to give a final optical density at 364nm (the excitation wavelength for DPH) of 0.15±0.02. The probe DPH was dissolved in formamide and added to these membranes at a final concentration of 0.003nmol l^{-1}. These concentrations of membranes and probe were selected to provide the optimal membrane:probe ratio, as determined in previous studies (E. E. Williams, personal communication). The membranes were incubated at 20˚C for at least 30min to allow probe equilibration. In some preparations, the membranes were pelleted and washed in 10mmol l^{-1} Hepes to remove BSA. However, the presence of BSA in the mitochondrial preparations did not affect the measured polarization values.

Cytochrome c *oxidase activities*

Cytochrome *c* oxidase (EC 1.9.3.1; ferrocytochrome *c* oxygen oxidoreductase) activities were measured using the protocol of Yonetani and Ray (1965). The assay medium contained 0.1mol l^{-1} Mes, pH6.0 at 20˚C, 1mmol l^{-1} EDTA and 50 μmol l^{-1} reduced cytochrome *c* (equine heart, type VI, Sigma). Reduced cytochrome *c* was prepared following the method of Hand and Somero (1983). Frozen mitochondria were defrosted, diluted 10-fold with 10mmol l^{-1} Hepes (pH7.4 at 20˚C) and immediately assayed at 5˚C increments from 20 to 65˚C. The oxidation of cytochrome *c* was observed

at 550nm. Assays were started by the addition of 10 μl of the diluted mitochondrial preparation, and initial rates of enzymatic activity were measured. Cytochrome *c* oxidase was unaffected by freezing when stored as frozen mitochondrial preparations, in agreement with previous studies (Dahlhoff *et al.* 1991).

Break temperature determinations

The Arrhenius break point (the temperature above which respiration or enzymatic activity drops off dramatically) was calculated by determining the two intersecting linear regressions that best fitted the data. The two lines of best fit were determined by maximizing their linear regression coefficients using Microsoft Excel for the Macintosh (Version 3.1; copyright 1991, Microsoft Co.). The break temperature was calculated by determining the intersection of these two lines. Each break temperature determination represents one individual.

Statistical determinations

All statistical tests were performed using Systat 5 for the Macintosh (Version 5.1; copyright 1991, Systat Inc.).

Results

Respiration of mitochondria from freshly collected abalone

The effects of measurement temperature on mitochondrial oxygen consumption exhibited a common pattern in all species: the rate of respiration increased up to a certain temperature, but any further increases in temperature led to a sharp reduction in respiration rate. This pattern is shown most clearly when the data are presented on Arrhenius plots (Fig. 1). The temperature at which the sharp change in slope was observed is termed the Arrhenius break temperature (ABT). For mitochondria from freshly collected abalone, the ABT correlated significantly with the habitat temperature of each species. There was a higher degree of correlation between ABT and capture temperature (Table 2) than between ABT and maximal habitat temperature (Table 1) (regressions not shown; ABT *versus* maximal temperature: $y=24.7+0.5x$; $r^2=0.704$; ABT *versus* capture temperature: $y=24.0+0.7x$; $r^2=0.855$). Therefore, the break temperatures of mitochondrial respiration from species living at warmer temperatures [*H. corregata* (pink) and *H. cracherodii* (black)] were significantly higher than for those of species from cool habitats [*H. rufesens* (red) and *H. k. kamtschatkana* (pinto)] (analysis of variance, ANOVA; $P=0.003$; Table 2). This pattern was observed for break temperatures calculated using both coupled and uncoupled (FCCP added) respiration rates. The addition of the respiratory uncoupler FCCP stimulated oxygen consumption in all cases (note the difference in scale between Fig. 1A and Fig. 1B), but there were no significant differences in break temperature between coupled and uncoupled rates (ANOVA; $P=0.654$). Routinely uncoupling mitochondria eliminates the possibility that the different responses of mitochondria to thermal stress may be caused, in part, by differential uncoupling (see Discussion).

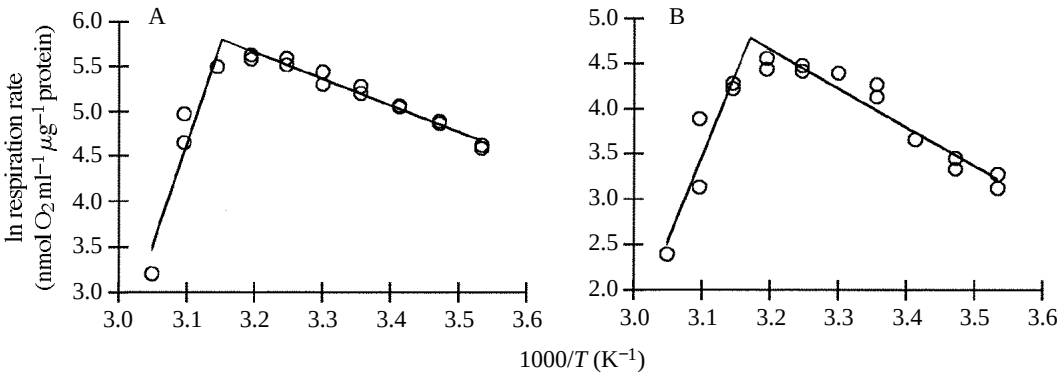

Fig. 1. Effects of temperature on the rates of oxygen consumption by abalone mitochondria, displayed as Arrhenius plots. Assay temperature increases towards the left. The Arrhenius break temperature (ABT, the temperature above which respiration decreases rapidly) was calculated as described in the text. Within a species, the break temperature of succinate-supported oxygen consumption for mitochondria uncoupled by the proton ionophore FCCP (A) was not significantly different from the ABT determined for mitochondria not treated with FCCP (B). Data shown are from one experiment (one individual) and are representative of ABT data reported in this study.

Fluidities of mitochondrial membranes from freshly collected abalone

Measurements of the steady-state fluorescence of the membrane probe DPH revealed differences in the fluidity of mitochondrial membranes from freshly collected abalone (Fig. 2). At all measurement temperatures, mitochondrial membranes from the species captured at the warmest temperature (pink and black abalone), were less fluid (as indicated by higher polarization values) than membranes from the two species living at cooler temperatures (red and pinto abalone). These data suggest that mitochondrial membranes of naturally occurring abalone have fluidities adapted for the temperature at which the animal is living, in agreement with homeoviscous theory. If the polarization at 20˚C is plotted as a function of both capture temperature and maximal habitat temperature (as was reported for mitochondrial respiration), there is a stronger correlation between ABT and capture temperature than between ABT and maximal habitat temperature (regressions not shown; ABT *versus* capture temperature: y=0.27+0.004x; r^2=0.983; ABT *versus* maximal temperature: y=0.25+0.004x; r^2=0.756).

Respiration of mitochondria from laboratory-acclimated abalone

The effect of temperature on mitochondrial function was measured after acclimation of abalone to their maximal, minimal and/or capture-habitat temperature. The length of time for thermal acclimation was determined by measuring the time course of warm-acclimation for green abalone (Fig. 3). These data show that acclimation of the ABT was complete within 14 days after the shift of water temperature. Studies of the time course of acclimation in vertebrates have shown that, although thermal acclimation occurs more slowly in response to lowered temperatures than for elevated temperatures, cold-acclimation of membrane composition and fluidity is complete within 21–36 days of the

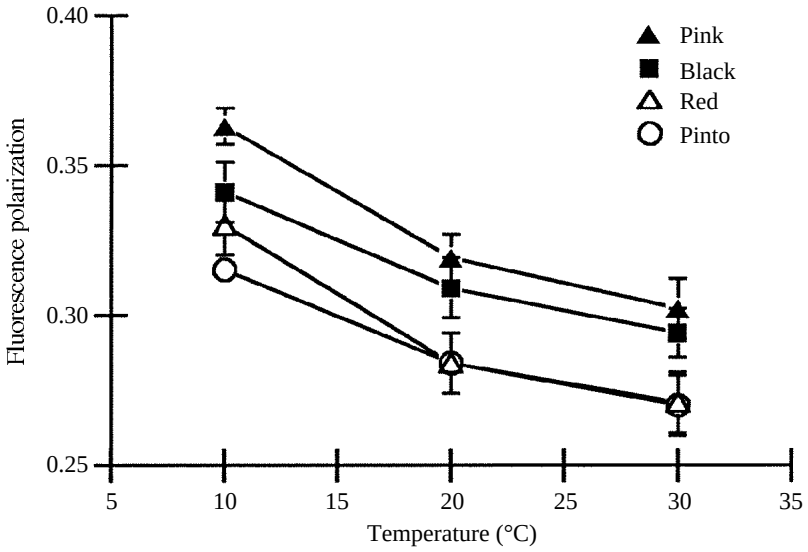

Fig. 2. The fluorescence polarization of DPH incorporated into mitochondrial membranes isolated from freshly collected abalone. Values are means ± 1 S.E.M. *N*=3 for all species.

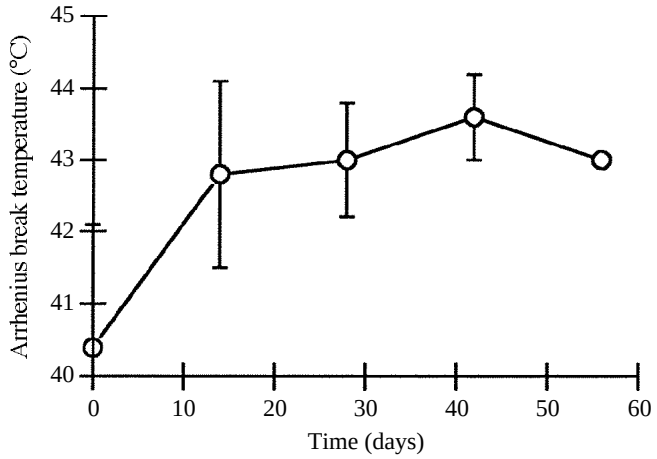

Fig. 3. The time course of acclimation of the Arrhenius break temperature of respiration of mitochondria isolated from green abalone acclimated from 20 to 26˚C. Individuals were placed in water at 26˚C after having been held at 20˚C for 30 days. Mitochondrial respiration was assayed at day 0 and every 2 weeks until 60 days of acclimation had been completed. Values are means ± 1 S.E.M. *N*=2 for all points except 60 days, where *N*=1 (because of a limited number of individuals).

initiation of cold acclimation (Cossins *et al.* 1977; Sellner and Hazel, 1982). It is therefore likely that the 42 days of acclimation allowed in the present study was adequate to reach the ‘end-point’ of both warm- and cold-acclimation.

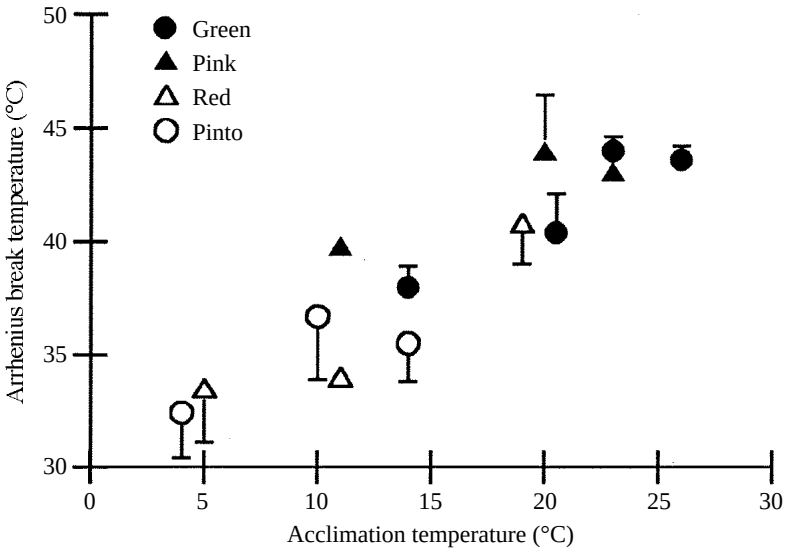

Fig. 4. The effect of acclimation temperature on the Arrhenius break temperature of mitochondrial respiration (y=29.50+0.586x; r^2=0.911). Values are means ±1 S.E.M. N=4 for all species and temperatures except for pink at 23˚C and pinto at 5˚C, where N=3. Overlapping data points (pinto 5˚C, red and pink 20˚C) have been offset by 1˚C for clarity.

The ABT of mitochondrial respiration correlated strongly with acclimation temperature when the results from four species of abalone were combined (Fig. 4; r^2=0.911). However, the ABT of oxygen consumption shifted in an adaptive manner to a much greater extent for mitochondria from green, pink and red abalone than it did for mitochondria from pinto abalone. Acclimation of abalone to their approximate capture temperatures did not result in significant shifts in the ABT of mitochondrial respiration when compared with the ABTs of mitochondria from freshly collected (or cultured) individuals (ANOVA; P=0.270 for green abalone; P=0.180 for pink abalone, P=0.270 for red abalone, and P=0.090 for pinto abalone).

Mitochondrial acclimation occurred over the range of temperatures at which each species is commonly found (Table 1). Specifically, the ABT of mitochondria from green abalone shifted from 38.0±0.9˚C for abalone held at 14˚C to 43.6±0.6˚C for individuals held at 23˚C (ANOVA; P=0.012). Similarly, the ABT of mitochondria from red abalone shifted from 33.5±2.4˚C at 5˚C to 40.8±1.8˚C at 20˚C (P=0.029), and the ABT of mitochondria from pink abalone shifted from 39.8±0.1˚C at 11˚C to 44.0±1.7˚C at 20˚C (P=0.010). The ABT of mitochondria from the stenothermal pinto abalone did not shift significantly (32.4±2.7˚C at 5˚C, 35.5±1.7˚C at 14˚C; P=0.090).

Fluidities of mitochondrial membranes from laboratory-acclimated abalone

At a given measurement temperature (20˚C), a significant correlation was found between acclimation temperature and the fluidity of mitochondrial membranes (Fig. 5: y=0.253+0.002x; r^2=0.600). Over the entire range of temperatures studied with the four

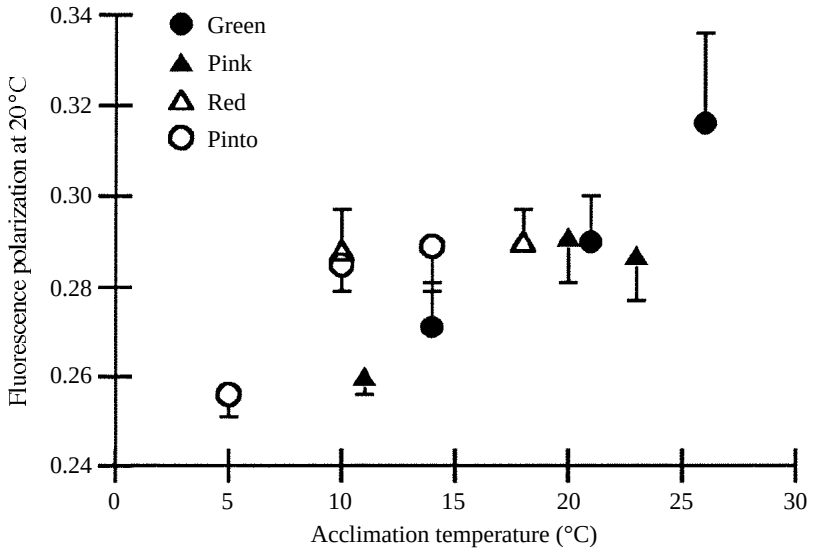

Fig. 5. The effect of acclimation temperature on the fluidity of mitochondrial membranes. There is an inverse correlation between mitochondrial membrane fluidity and acclimation temperature (y=0.253+0.002x; r^2=0.600). Values are means ± 1 S.E.M. N=3 for all species.

species, fluidity was inversely related to temperature of acclimation. However, as for the changes in the ABTs of mitochondrial respiration, the membrane fluidity changes occurred over the range of temperatures that each species commonly encounters; outside that range, thermal acclimation failed to alter fluidity significantly (Fig. 6). For example, green abalone commonly occur at temperatures between 18 and 23˚C. The fluidities of mitochondrial membranes from green abalone acclimated to 14˚C and 20˚C did not differ, but membranes from individuals acclimated to 26˚C were much less fluid than membranes from individuals acclimated to 20˚C (Fig. 6A). A similar trend was found for pink abalone, which are most common between 12 and 20˚C. Mitochondrial membranes from pink abalone acclimated to 20˚C were significantly less fluid than membranes from individuals acclimated to 11˚C (Fig. 6B). An increase in acclimation temperature to 23˚C did not induce further alterations in mitochondrial membrane fluidity. Adaptive alterations in membrane fluidity were observed for acclimation temperatures between 5 and 10˚C for membranes from pinto abalone, but not at 14˚C (Fig. 6D). Surprisingly, membranes from red abalone did not show temperature adaptive alterations in membrane fluidity (Fig. 6C).

Cytochrome c *oxidase activities of acclimated abalone*

Thermal acclimation shifted the Arrhenius break temperature of cytochrome *c* oxidase activity for mitochondria from green, pink and red abalone but not for mitochondria from pinto abalone (Fig. 7). The Arrhenius break temperature of enzymatic activity is less closely correlated with acclimation temperature than is the ABT of mitochondrial

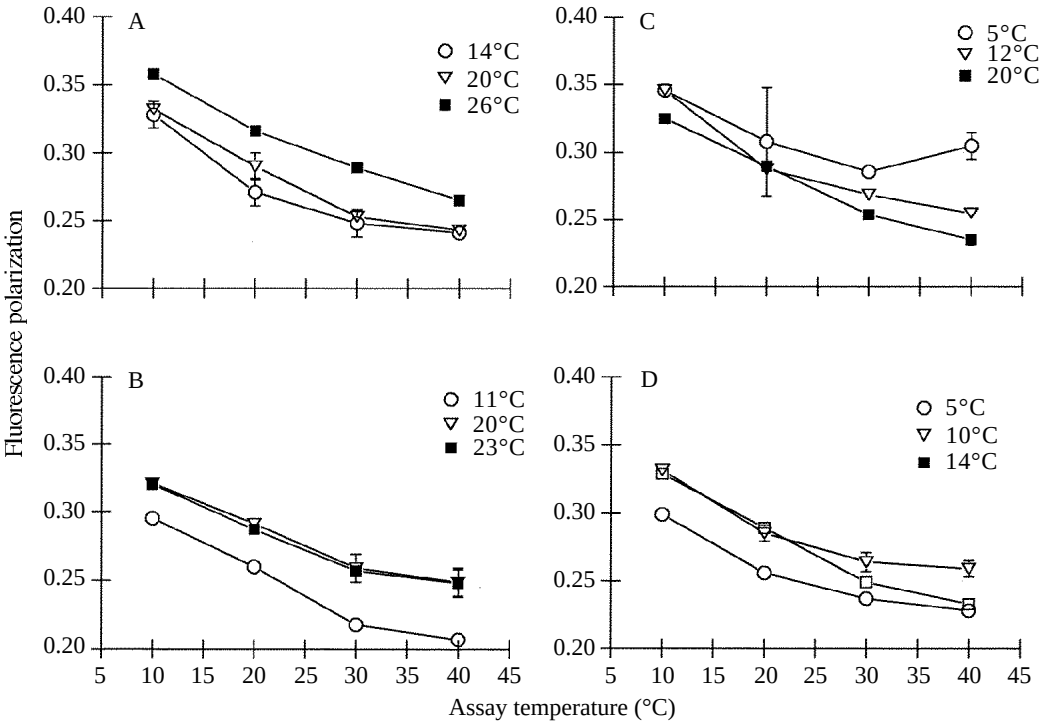

Fig. 6. The fluorescence polarization of DPH incorporated into mitochondrial membranes isolated from (A) green, (B) pink, (C) red and (D) pinto abalone acclimated to their maximal (filled squares), minimal (open circles) or capture-habitat (open triangles) temperatures. Values are means ± 1 S.E.M. *N*=3 in all cases.

respiration rate (r^2=0.422), but the overall pattern observed is similar in both cases (compare Fig. 5 with Fig. 7).

Discussion

Mitochondrial respiration measurements: effects of uncoupling

In this study, mitochondrial respiration was stimulated by the addition of exogenous substrate (succinate) and was then uncoupled using the proton ionophore carbonylcyanide-*p*-(trifluoromethyl)phenylhydrazone (FCCP). FCCP uncouples mitochondrial oxygen consumption from ATP production by destroying the electrochemical gradient generated by electron transport. Mitochondria that have been uncoupled should respire at a higher rate than coupled mitochondria.

We elected to study uncoupled mitochondria because we were unable to obtain consistent stimulation by ADP, i.e. a consistent degree of coupling, among different preparations of mitochondria. Therefore, to eliminate possible artefacts in the thermal responses that might have been caused by differences in the degree of coupling, we routinely studied respiration of FCCP-uncoupled mitochondria. However, we did not

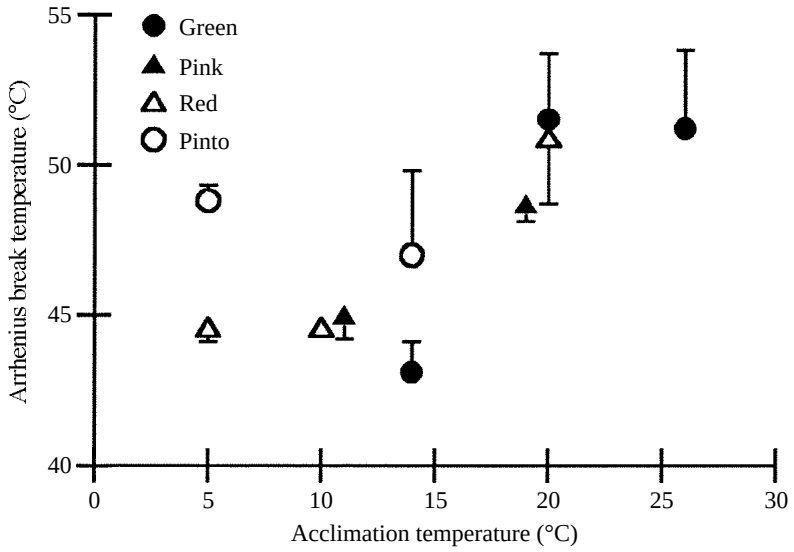

Fig. 7. The effect of acclimation temperature on the Arrhenius break temperature of cytochrome *c* oxidase from three species of abalone (y=43.62+0.282x; r^2=0.422). Values are means ± 1 S.E.M. N=3 for all species except pinto at 14°C, where N=2.

observe any difference in ABT between coupled and uncoupled mitochondria (Table 2). The only effects of uncoupling we observed were, first, the higher rate of oxygen consumption expected for uncoupled mitochondria and, second, a decrease in the temperature-dependence of respiration, which may reflect a shift in the rate-determining step of mitochondrial respiration with the addition of the uncoupler (Fig. 1).

Respiration and bulk membrane fluidities of mitochondria from freshly collected abalone

Mitochondria isolated from abalone collected at warm temperatures [*Haliotis corregata* (pink) and *H. cracherodii* (black)] are inactivated at significantly higher temperatures than mitochondria from species captured at cool habitat temperatures [*H. rufesens* (red) and *H. k. kamtschatkana* (pinto)]. The correlation between habitat temperature and the Arrhenius break temperature (ABT) of mitochondrial respiration found for freshly captured abalone is in agreement with studies of mitochondria from deep-sea hydrothermal vent invertebrates (Dahlhoff *et al.* 1991). These comparative studies show that ABTs are strong indicators of temperature adaptation. In previous studies, the maximal habitat temperature has been shown to be the most powerful predictor of adaptation temperature (Coppes and Somero, 1990; Dahlhoff and Somero, 1991; Dahlhoff *et al.* 1991). In the present study, however, the ABT of mitochondrial respiration correlates more closely with capture temperature (P=0.855) than with maximal habitat temperature (P=0.704). This result suggests acclimation of some aspect(s) of mitochondrial function in response to changing environmental factors.

Differences observed in the fluidities of mitochondrial membranes of freshly

collected abalone are consistent with homeoviscous theory and are in agreement with previous studies of adaptive differences in membrane fluidities of teleosts and other vertebrates (Cossins and Bowler, 1987; Hazel and Williams, 1990). The present study is one of the first examples of homeoviscous adaptation to temperature of membrane lipids of closely related species living in different thermal habitats. The higher degree of correlation between capture temperature and membrane fluidity at 20˚C (P=0.983) than between maximal habitat temperature and membrane fluidity (P=0.756) suggests that, as for the thermal resistance of mitochondrial oxygen consumption, mitochondrial membranes from freshly collected specimens are acclimatized to variations in habitat temperature.

Effects of thermal acclimation on mitochondrial respiration and bulk membrane fluidities

Differences observed in the effects of capture temperature on the thermal inactivation of mitochondrial respiration and the fluidity of mitochondrial membranes may be the result of non-acclimatable (fixed) traits, thermally plastic (acclimatable) processes, or a combination of these factors. Acclimation of abalone to temperatures at the extremes of the ranges in which they are most abundant resulted in adaptive shifts of membrane fluidities and/or the resistance of mitochondrial respiration to thermal stress in all species, but further acclimation to temperatures at the extremes of each species' full biogeographical range did not induce further acclimation. This is the first evidence for differences in the ability of closely related species of marine invertebrates to acclimate thermally and suggests that both plastic and non-acclimatable (see Dahlhoff and Somero, 1993) physiological traits are important for determining abalone distribution.

The effects of acclimation temperature on the Arrhenius break temperature of mitochondrial respiration show that, for the eurythermal green, pink and red abalone, acclimation to maximal habitat temperatures resulted in a significant increase in the ABT of mitochondrial respiration relative to the ABT of respiration from individuals acclimated to their coolest habitat temperatures. However, the ABT of mitochondrial respiration of the stenothermal pinto abalone did not shift significantly in specimens acclimated to elevated temperatures. Therefore, differences in thermal plasticities of mitochondrial oxygen consumption appear to correlate with the habitat temperature range of each species.

The bulk fluidities of mitochondrial membranes were altered in an adaptive manner in green, pink and pinto abalone. Like mitochondrial respiration changes, membrane fluidity changes only occurred over the range of temperatures at which each species is most abundant; outside this range, thermal acclimation failed to alter fluidity significantly. This observation suggests that the range of temperatures in which a given species can live successfully is determined, in part, by the ability to alter biochemical structures such as membranes. For example, green abalone live in the mid-intertidal zone in Baja California and may therefore experience daily wide swings in habitat temperature. The fluidities of mitochondrial membranes isolated from green abalone acclimated to 20˚C are similar to the fluidities of membranes from pink abalone acclimated to 20˚C (Figs 5 and 6). However, acclimation of pink abalone to their maximal temperature of 23˚C did not

further alter mitochondrial membrane fluidity, whereas green abalone membranes acclimated to 26˚C showed a further reduction in fluidity.

At all measurement temperatures, the fluidity of mitochondrial membranes of pinto abalone acclimated to 5˚C was greater than the fluidity of membranes from individuals acclimated to 10˚C. As seen for the pink abalone, acclimation to the pinto abalone's maximal habitat temperature (14˚C) did not result in further decreases in membrane fluidity. These results support the observation that pinto abalone prefer temperatures below 12˚C (Paul and Paul, 1980).

It is unclear why membranes from red abalone acclimated to cool habitat temperatures (5 and 10˚C) are not more fluid than those of specimens acclimated to 20˚C. In view of this observation, some discussion of the linkage between the functional and structural aspects of membrane-associated processes (such as respiration) is warranted. Previous studies of enzymatic systems associated with membranes have shown that alterations of the fluidity or lipid composition of a given membrane in response to different acclimation temperatures may (Hazel, 1972; Gibbs and Somero, 1990) or may not (van den Thillart and Modderkolk, 1978; Raynard and Cossins, 1991; Trigari *et al.* 1992) correlate with alterations in enzymatic or respiratory function. The lack of a strong correlation between fluidity and the function of membrane-associated processes in these cases has been explained by hypothesizing that the lipids most closely associated with membrane-bound proteins (so-called annular lipids), rather than 'bulk' lipids, are most responsible for the proper functioning of membrane-associated proteins (Vik and Capaldi, 1977; van den Thillart and Modderkolk, 1978; O'Brien *et al.* 1991).

The observation that mitochondrial respiration and cytochrome *c* oxidase activity of red abalone, but not their membrane fluidity, acclimate suggests that caution should be exercised in implying that changes in fluidity cause adaptive alterations in mitochondrial respiration. Rather, structural changes (as indexed by membrane fluidity) and functional alterations (as indexed by respiratory measurements) of mitochondria should be considered as two somewhat independent physiological indicators of adaptation or acclimation to temperature changes that may be, but are not necessarily, related.

Studies of membranes from species living at, or acclimated to, different habitat temperatures have revealed that, although there is often a strong correlation between membrane fluidity and habitat or acclimation temperature, there is not a complete conservation of fluidity at normal body temperatures. That is, the efficacy of homeoviscous adaptation is usually less than 100% (Cossins, 1983). There is no general explanation for why homeoviscous adaptation is not complete, but many factors may be involved, including heterogeneities in the bilayer and functional limitations of annular-lipid/protein interactions (Hazel and Williams, 1990). In the present study, the efficacy of homeoviscous adaptation for acclimated mitochondrial membranes was approximately 90%. This result is not surprising, because a high degree of efficacy has been observed for mitochondrial membranes from teleosts (Hazel and Williams, 1990).

Acclimation of the thermal resistance of cytochrome c *oxidase*

The acclimation pattern for thermal inactivation of cytochrome *c* oxidase is similar to that observed for mitochondrial respiration, although the correlation with acclimation

temperature is not as strong as it is with mitochondrial oxygen consumption (compare Fig. 5 with Fig. 7). The observation that acclimation temperature shifts the thermal resistance of cytochrome *c* oxidase activity in an apparently adaptive manner in mitochondria isolated from green, pink and red abalone suggests that some aspect of cytochrome *c* oxidase function shifts with acclimation temperature.

As discussed in the previous section, one explanation for adaptive shifts in cytochrome *c* oxidase activity is that annular lipid substitution may result in an increase of the thermal resistance of cytochrome *c* oxidase. Additionally, previous studies suggest that alterations in overall membrane lipid composition may influence the activity and thermal stability of membrane-associated enzymes (Hazel, 1972; Cossins *et al.* 1981). For example, Cossins *et al.* (1981) showed that thermal acclimation induced coupled shifts in membrane fluidity and thermal stability of Na^+/K^+-ATPase from goldfish synaptosomes. Other evidence suggests that the lipid bilayer offers some resistance to the conformational changes required for enzymatic catalysis, and the magnitude of resistance can be modified by alterations in lipid composition (Sinensky *et al.* 1979; Harris, 1985). Like these examples, the changes in the thermal tolerance of cytochrome *c* oxidase with thermal acclimation observed in the present study may be a direct result of alterations in membrane composition, fluidity, or both.

Ecological and evolutionary implications

The ability of the eurythermal green, pink and red abalone to acclimate the ABT of mitochondrial respiration or membrane fluidity may be due, in part, to adaptations to a habitat where the temperature varies either diurnally or seasonally. For example, red and green abalone are distributed into the intertidal zone over some part of their geographical range, whereas the stenothermal pinto abalone is distributed almost exclusively below the mean low tide level (Paul and Paul, 1980). Although pink abalone are not found in the intertidal zone at low latitudes, this distribution may be misleading. Historically, the distributions of pink abalone suggest that this species occurred intertidally in Baja California before humans decimated their population. Therefore, because pink abalone appear to be adapted to function at elevated temperatures almost as well as are green abalone, pink abalone are probably not limited to the subtidal zone because of water temperature. Rather, non-physiological factors (food preference, predation, competition with other abalone species) are probably more important determinants of pink abalone distribution at these lower latitudes.

Previous studies of abalone malate dehydrogenases suggest that red abalone are adapted to function optimally at cooler temperatures than are pink abalone (Dahlhoff and Somero, 1993). However, red and pink abalone co-occur from Point Conception to northern Baja California (Owen *et al.* 1971). This distribution, along with adaptive shifts in the thermal tolerance of mitochondrial respiration and cytochrome *c* oxidase of red abalone, provides evidence that red abalone may have evolved adaptations to acclimate some physiological processes to elevated temperatures since their divergence from the common ancestor they shared with pinto abalone. These adaptations would allow red abalone to exploit warmer habitats than pinto abalone. The differences in biogeographical distribution between red and pinto abalone suggest that this hypothesis is not entirely unreasonable.

In conclusion, the data presented in this study provide evidence that both acclimatable and non-acclimatable differences in mitochondrial structure and function may influence the distributions of abalone species. Furthermore, the observation that mitochondrial respiratory function and membrane fluidity acclimated only over the range of temperatures at which each species is most common suggests that thermal plasticity is important in defining the range of habitat temperatures over which a species can thrive.

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