Heterologous acclimation: a novel approach to the study of thermal acclimation in the crab Cancer pagurus

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The ability of ectothermic animals to maintain function in environments that are subject to diurnal and seasonal fluctuations of temperature has been extensively studied. Approaches used have concerned measurements of compensatory or adaptive changes in animals that have experienced natural fluctuations of environmental parameters (acclimatization) or, more usually, studies with a single laboratory-controlled parameter (acclimation). In this way, an extensive catalogue of thermal acclimation and acclimatization responses has been demonstrated (5), particularly for aquatic species. For example, at the level of the organism such compensatory responses have been shown to be adaptive in thermal tolerance (10), thermal preference (3), locomotion (28), and metabolism (14). More recently attention has focused on determining the adaptive responses that explain those adaptive responses. With respect to recently attention has focused on determining the ability of ectothermic animals to maintain function in environments that are subject to diurnal and seasonal fluctuations of temperature has been extensively studied. Approaches used have concerned measurements of compensatory or adaptive changes in animals that have experienced natural fluctuations of environmental parameters (acclimatization) or, more usually, studies with a single laboratory-controlled parameter (acclimation). In this way, an extensive catalogue of thermal acclimation and acclimatization responses has been demonstrated (5), particularly for aquatic species. For example, at the level of the organism such compensatory responses have been shown to be adaptive in thermal tolerance (10), thermal preference (3), locomotion (28), and metabolism (14). More recently attention has focused on determining the adaptive responses that explain those adaptive responses. With respect to metabolism, thermal acclimation can alter metabolite flow through branch points where pathways compete for 1) the same substrate (14), 2) increased mitochondrial enzyme activity (12), and 3) the volume fraction of muscle occupied by mitochondria (6). These changes have been interpreted as compensatory responses to permit enhanced performance at lower environmental temperatures. Acclimation effects have also been demonstrated on the contractile properties of fish muscle (1) and on Ca\(^{2+}\) ATPase activity (38) that clearly would contribute to the observed responses on swimming performance.

The mechanisms involved in bringing about such hierarchical acclimation responses have in recent times focused on three approaches. First, there is the remodeling of the membrane lipid (15). Implicit in this approach is that the structural adjustments in lipid composition change membrane physical state in a manner that compensates for the direct impact of the temperature change. The adaptive significance is to produce a constancy of membrane function (4). Second, changes occur in the concentrations of substrates, cofactors, and enzymes (23). These kinetic responses are again argued to be adaptive in that they compensate for the direct effect of the temperature change on enzyme activities. Third, the differential expression of some protein isoforms has been described to occur during thermal acclimation and acclimatization (21, 39), the implication being that the expression of the isoform most appropriate for the prevailing thermal conditions was adaptive (31). More recently, interest has focused on temperature-induced expression of specific enzymes. Cold-induced expression of a desaturase from carp liver has been reported (35). The thrust of this approach was to relate the temperature-induced expression of the enzyme with changes in membrane lipid saturation to shed light on the cellular mechanisms that report the temperature change.

Implicit in those mechanistic approaches is that acclimation responses occur at the level of the cell. Evidence from fish cell lines shows that isolated cells are capable of acclimation. Responses have been reported in membrane “fluidity” (36), the specific activities of some enzymes (19), microtubule stability (37), and heat resistance (29) consistent with those seen in tissues from whole organisms. However, a possible hormonal requirement was proposed for protein synthesis in cultured fish hepatocytes (24). This raises the interesting question of whether acclimation responses of an organism are a simple summation of individual cellular responses or whether they require an involvement of central control systems (2). Opinion exists that suggests that acclimation may be controlled systemically either directly or indirectly (19, 20, 25).

The proposed study seeks to address this question with a novel technique. Crabs were maintained such that different regions of the same animal were held...
simultaneously at different (heterologous) temperatures. This technique has been used previously in relatively few studies (7, 26). These earlier studies used fish, and the metabolic responses investigated suggested that the central nervous system (CNS) played a hierarchical role. Those experiments with fish were difficult to interpret for two reasons: first, because the fish were divided anterior-posterior and second, because of the variation in the mass of tissue exposed to the different temperatures (7). In the present study, crabs were maintained at the heterologous temperatures with an offset lateral separation of the body. This allowed a direct comparison to be made of the muscles, from the legs of the same crab, that had experienced different thermal histories. Furthermore, it enabled these comparisons to be related to the temperature experienced by the CNS and eye-stalk hormonal system. The neuromuscular system was chosen for study because it has been shown to be responsive to thermal acclimation in a variety of decapod crustaceans (13, 16, 33). The efficacy of the heterologous temperature paradigm was established by comparison with information obtained with similar electrophysiological measurements on crab leg muscles in which the whole body was uniformly held for acclimation at 8 or 22°C (homologous acclimation).

MATERIALS AND METHODS

Condition for homologous acclimation. Cancer pagurus were fed after capture and held in filtered seawater at 8°C. Crabs were then held at 8 ± 0.5°C for a further 17 days before electrophysiological testing. Other crabs were transferred to increased temperature in steps, over a period of 17 days, to 22°C. Crabs were held at that temperature for a further 2 wk before use (32).

Immobilized controls. Crabs were placed in the heterologous apparatus (Fig. 1), and both compartments either were maintained at 8 ± 0.5°C for 2 wk or were exposed to increasing temperatures up to 22°C and then maintained for a further 2 wk at 22 ± 0.5°C. These controls, compared with free-moving homologous acclimated crabs, identified any effects of immobilization on the selected neurophysiological parameters.

Heterologous acclimation. The same temperatures were selected for heterologous acclimation as those used in the control homologous free and immobilized acclimation experiments, i.e., 8 and 22°C. Crabs of appropriate size (10-cm-width carapace) were chilled to facilitate handling. The crab was then fitted into the apparatus so that the rubber partition diaphragms separated the body of the animal longitudinally into right and left sides. The diaphragm was positioned off-center so that it was between a cheliped and the mouth parts (Fig. 1); this arrangement isolated a three-quarter body section, including the CNS, both eye-stalks, and ipsilateral legs at one acclimation temperature, whereas the contralateral quarter was isolated at the other acclimation temperature. The animal was then clamped securely in the apparatus.

Two heterologous temperature protocols were arranged as follows: 1) the larger body portion at 8°C and the smaller part at 22°C, a condition designated 8CN/8Leg and 8CN/22Leg; and 2) the larger body portion at 22°C and the smaller part at 8°C, a condition designated 22CN/22Leg and 22CN/8Leg, where CN refers to central temperature and Leg refers to leg temperature.

Comparisons could then be made of the extent of the acclimation responses between legs of opposite sides in the same animals.

The conductivity of the seawater was monitored and maintained to ensure that crabs did not experience changes in osmotic conditions. Crabs were not fed during the 2-wk acclimation period. In a series of control measurements of tissue temperatures with implanted thermocouples in crabs incubated under heterologous temperature conditions, the CNS temperature was 19.0 ± 0.16 or 9.9 ± 0.096°C when the external temperatures were 21.8 ± 0.06 or 8.3 ± 0.15°C, respectively. In effect, the difference in CNS temperatures achieved was 9.1°C, which was a smaller difference than was found between the water baths (13.5 ± 0.17°C) in these control experiments. These acclimation temperature differences were also smaller than those used for homologous temperature acclimation, which was 14°C (8 ± 0.5 vs. 22 ± 0.5°C). The leg muscle temperatures were the same as bath
temperature and so were the same as for the homologous temperature-acclimated crabs.

Dissection and electrophysiology. After acclimation, a walking leg was automotized, and the tonic motor axon to the dactylopodite closer muscle was identified within the propodite article. Dactylopodite closer muscle fibers were then revealed in the propodite article by removal of the overlying carapace. Electrophysiological recordings of resting potential (RP), single excitatory junction potential (EJP), and facilitation were made from dactylopodite closer muscle fibers (types I and II, Ref. 27) with intracellular microelectrodes with conventional recording techniques. The single tonic axon to the closer muscle was selected by nerve splitting and stimulation threshold. Care was taken to ensure that the common inhibitor axon innervating the closer muscle was not stimulated. The experimental temperature range (6–26°C) was controlled by a Peltier junction. In most experiments, the temperature characteristics of measured parameters were determined at selected intervals during a ramp change (0.4 °C/min) in experimental temperature. Sixteen records were taken from each closer muscle fiber at each selected interval (–1–2 °C) within the experimental temperature range and were averaged and analyzed later with the SCAN software package (J. Dempster, Univ. of Strathclyde). Tissues were bathed in crab saline [composition (in mM): 470 NaCl, 20 CaCl₂, 10 MgCl₂·6H₂O, 8 KCl, 10 HEPES acid, pH 7.4 (32)].

RESULTS

Muscle membrane RP. Figure 2 shows the data for RP measurements for dactylopodite closer muscle fibers of Cancer pagurus homologously maintained at temperatures of 8 and 22°C. RP hyperpolarized with increasing experimental temperature in both free and immobilized animals. In both groups, leg muscle RP from cold-maintained animals was significantly hyperpolarized over the whole experimental temperature range compared with those from warm-maintained crabs. In 8°C-maintained crabs, immobilization did not result in significant difference in the RP dependency on temperature; the slope of the regression was −1.29 ± 0.16 and −1.08 ± 0.06 mV/°C for the free (n = 14) and immobilized (n = 16) crabs, respectively. However, immobilization had an effect in the 22°C-maintained crabs; the slope of the regression fell from −1.05 ± 0.061 to −0.76 ± 0.078 mV/°C in the free (n = 16) compared with the immobilized (n = 10) crabs, respectively (P < 0.05). The efficacy of the acclimation response was 69% for the free compared with 104.3% for the immobilized group.

Figures 3 and 4 show the effect of temperature on the muscle membrane RP of heterologously maintained crabs. Muscle membranes hyperpolarized in a linear fashion with increasing experimental temperature. In the first experiment in which the CNS was maintained at 8°C (Fig. 3), a clear acclimation shift of leg muscle RP occurred between the 8CN/8Leg and 8CN/22Leg conditions. The muscle membranes from the legs of the ipsilateral side (8°C) were significantly hyperpolarized compared with the legs on the contralateral side (22°C), and the efficacy of the acclimation was 72%. Figure 4 shows similar data for 22CN/22Leg and 22CN/8Leg conditions. In these experiments also, maintenance of the CNS at the higher temperature did not prevent the acclimation response of leg muscle RP as shown by the displacement of the RP vs. temperature relationship, although the efficacy of the acclimation was reduced to 48%. The slopes of the regression relationship for RP vs. temperature were −0.98 ± 0.07 (8CN/8Leg), −0.92 ± 0.08 (8CN/22Leg), −1.01 ± 0.07 (22CN/8Leg), and −0.92 ± 0.04 (22CN/22Leg) mV/°C and were not significantly different.

EJP amplitude. Figure 5 shows the amplitudes of walking leg EJP obtained over the experimental range of temperatures for both free and immobilized crabs.
homologously held at 8°C. In these animals, the EJP amplitude was largest at an average of 1.50 mV over the range 6–13°C and was reduced progressively in amplitude to approximately 25% of this value at 25°C. The amplitude of the EJP from immobilized crabs showed a similar pattern of change with experimental temperature (Fig. 5) as that of the unrestrained crabs, but over most of the temperature range (6–17°C) the EJPs obtained from immobilized animals were significantly larger. A different pattern of response of EJP amplitudes to acute temperature change was obtained from the legs of 22°C homologously maintained crabs. Relatively little change in EJP amplitude occurred between 11 and 24°C. No significant differences in EJP amplitudes were observed between free and immobilized crabs acclimated at 22°C. Clear differences in EJP amplitudes vs. temperature relationships were observed for different acclimation temperatures. In both free and immobilized crabs, EJP amplitudes were larger for 8°C-maintained compared with 22°C-maintained crabs below acute experimental temperatures of 19–20°C but smaller above these values (Fig. 5).

The dependency of EJP amplitude on temperature for heterologously maintained crabs is shown in Figs. 6 and 7. Examination of Figs. 6 and 7 shows that a similar pattern of change in EJP amplitudes vs. temperature was obtained from both legs irrespective of whether the temperature of the CNS was maintained at 8 or 22°C. Thus EJP amplitudes, as in the homologously maintained crabs, were larger for 8CN/8Leg and 22CN/8Leg over the temperature range 5–19°C compared with EJP amplitudes of 8CN/22Leg and 22CN/22Leg. Above experimental temperatures of 19°C, EJP amplitudes of 8CN/22Leg and 22CN/22Leg were maintained to a higher temperature than those from 8CN/8Leg and 22CN/8Leg. These differences were qualitatively independent of CNS acclimation temperatures. The acclimation responses of the 8CN/8Leg and 22CN/8Leg were similar to those obtained from 8°C homologously maintained crabs (Fig. 5) in that the highest values were obtained between 6 and 15°C. EJPs progressively declined with increased experimental temperature. The
pattern of response obtained from 8CN/22Leg and 22CN/22Leg of the two different heterologous temperature conditions was similar and was in agreement with the data from 22°C homologously maintained crabs.

Facilitation and latent period. The acute temperature dependency of facilitation of the EJP and its latent period from nerve stimulation in free and immobilized 8- and 22°C-maintained crabs was also measured. However, no significant differences were found between any of the groups of crabs maintained at 8 and 22°C. Nor were significant differences found in EJP facilitation or latent period with acute experimental temperature change, the shift of this relationship with long-term temperature change appears to be adaptive. In unrestrained crabs, the RP vs. temperature relationship is displaced to higher temperatures on maintenance at 22°C, a shift that partially compensates for the hyperpolarizing effect of an acute increase in temperature. The extent of this compensation in Cancer pagurus leg muscle RP was 69% in crabs maintained at 8 or 22°C compared with 72% in heterologously maintained crabs in which the central temperature was ~10°C. The fact that peripheral acclimation responses of leg muscle RP occur to a similar extent in heterologous temperature conditions demonstrates that compensatory peripheral responses to temperature can proceed independent of the central temperature. With central components maintained at higher temperatures (~19°C), the acclimation response between 22CN/22Leg and 22CN/8Leg muscle RP was only 48%; that is, the compensatory effect was reduced. This difference in the extent of compensation suggests some modulatory role in the attainment of peripheral tissue acclimation when CNS-hormonal systems were maintained at the higher acclimation temperature. Immobilization did affect the RP vs. temperature relationship in 22°C-maintained controls only. This immobilization effect was not evident in heterologously maintained crabs because the slopes of the relationships were not different from those obtained for free crabs.

In walking leg muscle fibers of 8°C-maintained crabs, EJP amplitudes decreased with increasing acute experimental temperature, whereas those recorded from 22°C-maintained walking leg muscles were relatively constant over much of the same temperature range. EJP amplitudes were consistently larger in legs maintained at 22°C homologously maintained crabs.

Crab walking leg muscle fibers exhibited hyperpolarizing RP with increasing experimental temperature, irrespective of long-term maintenance temperature of walking legs or the CNS. Hyperpolarizing RPs have been reported in other crustacean muscle fibers (8, 18, 34, 40). However, the biphasic, nonlinear characteristic seen in some other crustacean muscles (17, 40) was not evident in Cancer pagurus drier muscle fibers used in this study. The RP dependency on temperature of all acclimation groups was significantly greater than the value predicted by the Nernst equation (0.3163 mV/°C). The temperature dependency of RP change was considered because of changes in Na⁺-K⁺-ATPase activity (25, 40), but temperature-induced changes in Na⁺ and/or K⁺ permeability may also be significant (25). In one series of experiments with 8°C-maintained crabs, muscle fibers were incubated with ouabain, which reduced the temperature dependency of the RP from 1.34 to 0.4065 mV/°C. This clearly supports the view that all but 22% of the RP change with temperature can be accounted for by changes in Na⁺-K⁺-ATPase activity (40). The 22% discrepancy might be accounted for by ATP-independent processes, such as changes in Na⁺ and/or K⁺ permeability (25). Acclimation shifts of RP with temperature have been observed in a number of other nerve and muscle preparations (22). Whatever the mechanism linking change in RP with acute temperature change, the shift of this relationship with long-term temperature change appears to be adaptive.
at 8°C over the acute experimental temperature range between 6 and 19°C. The pattern of responses described is not essentially different whether the crabs were maintained under homologous or heterologous temperature conditions. Decreasing EJP amplitudes with increasing experimental temperature have been reported from Procambalurus darkii maintained at 10°C (8) and for 12°C-acclimated Astacus leptodactylus (13). Increased EJP amplitudes at warmer experimental temperatures have been reported in 25°C-acclimated Astacus leptodactylus (13), 21°C-acclimated Pachygrapsus crassipes (34), and Ocypode ceratophthalma, which generated maximal EJP amplitudes over 22–28°C when acclimated to 26–27.5°C (9).

Muscle tension with neuronal stimulation declines with warming in crustacean neuromuscular preparations (8, 13). Stephens (33) suggested that this might result from the combined effects of 1) RP hyperpolarization moving the membrane potential away from the excitation-contraction threshold, 2) reduction of the EJP amplitude, and 3) decline in the EJP time course. Changes observed in Cancer pagarus muscle RP and EJP amplitude in response to maintenance at warmer temperatures would largely offset these effects. Unlike EJP amplitudes in Pachygrapsus crassipes (34), however, facilitation did not show acclimation responses with temperature that might contribute to maintenance of muscle function. However, in Cancer pagarus we have shown that facilitation only increased at temperature extremes, a change that was not responsive to thermal history.

The differences in EJP amplitudes in response to direct temperature changes recorded in the present study on Cancer pagarus were dependent on the local leg muscle acclimation temperature and were relatively unaffected by temperature experienced by the CNS-hormonal systems. Immobilization resulted in an increase in EJP amplitude most evident at lower experimental temperatures in animals immobilized and acclimated to 8°C (Fig. 5). In immobilized animals maintained at 22°C, EJP amplitudes were not increased at the lower range of experimental temperatures. The different patterns of response in EJP amplitudes to temperature, shown in the 8 and 22°C homologously maintained preparations, were also evident in both heterologous temperature conditions. Here the pattern of the response was determined by the leg temperature and not the CNS temperature.

The evidence for a central influence in thermal acclimation is scant and in some cases inconclusive. A governing CNS influence could not be ruled out from work on trout, but no central influence affected trout critical thermal maxima (7). The acclimation status in fiddler crabs (Uca pugilator) has been reported to be influenced by hormones; eye-stalk extracts from 25°C-acclimated crabs decreased respiration when injected into 15°C-acclimated animals, whereas extracts from 15°C-acclimated crabs significantly increased respiration in 25°C-acclimated crabs (30). This was interpreted as the response to two antagonistically acting hormones on crab metabolic rates. A central hormonal factor that overrides local thermal effects on catfish hepatocytes has also been observed (25). Furthermore, different levels of spinal cord tonic discharges were correlated with persistent changes in muscle metabolism, suggesting that peripheral temperature acclimation was based on adaptive changes in the nervous system (20).

**Perspectives**

The heterologous temperature paradigm adopted can be used to identify the contributions made by the CNS-hormonal systems to acclimatory responses. This is important because seasonal acclimatization (as opposed to laboratory acclimation) to temperature is likely to be accompanied by responses to changes in a suite of other environmental factors. Some of these factors are likely to be transduced through the CNS alone and not have a direct influence on other tissues (e.g., photoperiod). Thus the heterologous thermal procedure will enable an assessment of the part played by these other factors in the complex, integrated processes of seasonal acclimatization to environmental change.

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