No evidence for homeoviscous adaptation in intertidal snails: analysis of membrane fluidity during thermal acclimation, thermal acclimatization, and across thermal microhabitats

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Abstract    Many eurythermal organisms alter composition of their membranes to counter perturbing effects of environmental temperature variation on membrane fluidity, a process known as homeoviscous adaptation. Marine intertidal gastropods experience uniquely large thermal excursions that challenge the functional integrity of their membranes on tidal and seasonal timescales. This study measured and compared membrane fluidity in marine intertidal snail species under three scenarios: (1) laboratory thermal acclimation, (2) thermal acclimatization during a hot midday low tide, and (3) thermal acclimatization across the vertical intertidal zone gradient in temperature. For each scenario, we used fluorescence polarization of the membrane probe DPH to measure membrane fluidity in individual samples of gill and mantle tissue. A four-week thermal acclimation of *Tegula funebralis* to 5, 15, and 25°C did not induce differences in membrane fluidity. *Littorina keenae* sampled from two thermal microhabitats at the beginning and end of a hot midday low tide exhibited no significant differences in membrane fluidity, either as a function of time of day or as a function of thermal microhabitat, despite changes in body temperature up to 24°C within 8 h. Membrane fluidities of a diverse group of snails collected from high, middle, and low vertical regions of the intertidal zone varied among species but did not correlate with thermal microhabitat. Our data suggest intertidal gastropod snails do not exhibit homeoviscous adaptation of gill and mantle membranes. We discuss possible alternatives for how these organisms counter thermal excursions characteristic of the marine intertidal zone.

Introduction

Biological membranes are structurally and functionally essential to all living organisms and are particularly sensitive to temperature variation because of the effect temperature has on the physical properties of lipids (Cossins and Prosser 1978; Hazel and Williams 1990). To function properly, membranes must exist in a state of fluidity maintained primarily by hydrophobic, van der Waals, and electrostatic interactions among the non-polar acyl-chain regions of phospholipids that compose the membrane bilayer. Both increases in temperature, which typically result in greater membrane fluidity, and decreases in temperature, which lead to greater membrane rigidity, can result in membrane dysfunction (Harris 1985; Cossins et al. 1986; Williams and Hazel 1992). Poikilotherms often compensate for the thermal sensitivity of their membranes by modifying membrane composition through changes in phospholipid head groups, acyl-chain length, acyl-chain saturation, and sterol content (Cossins and Prosser 1978; Hazel 1984). The adaptive reordering of membrane composition to conserve a biologically optimal range for membrane fluidity is referred to as homeoviscous adaptation (HVA) (Sinensky 1974; Hazel and Williams 1990; Hazel et al. 1992; Hazel 1995).
and has been reported in a range of organisms and tissues (Sinensky 1974; Cossins 1983; Behan-Martin et al. 1993; Logue et al. 2000; Overgaard et al. 2008).

A relatively straightforward approach to assess membrane fluidity, or more precisely membrane packing and order, is to use monochromatic polarized light to measure the anisotropy of fluorescent hydrophilic membrane probes (e.g. 1,6-diphenyl-1,3,5-hexatriene (DPH)) embedded in lipid bilayers (Pottel et al. 1983). DPH fluorescence anisotropy is inversely proportional to fluidity and thus decreases with increasing temperature as DPH molecules embedded in highly fluid membranes have increased rotational freedom, and thus lower anisotropy, than do DPH molecules in highly ordered low fluidity membranes (Hazel and Williams 1990). In HVA modifications to membranes (as described above), the mobility of embedded DPH is altered (Sinensky 1974; Pottel et al. 1983; Hazel and Williams 1990). The net result is that DPH anisotropy is similar at adaptation/acclimation temperatures across organisms with a wide range of body temperatures (Logue et al. 2000), but at a common measurement temperature, DPH anisotropy will be higher in lower fluidity membranes from warm-adapted or acclimated organisms than higher fluidity membranes from cold-adapted or acclimated organisms (Logue et al. 2000, Overgaard et al. 2008).

Marine intertidal poikilotherms routinely face extreme variation in habitat temperature on both a tidal and a seasonal basis (Williams and Somero 1996; Tomanek and Somero 1999; Helmhuth et al. 2002, Finke et al. 2007; Stillman and Tagmount 2009) and thus are expected to possess robust mechanisms for conserving membrane order during thermal excursions. In fact, both intertidal and subtidal marine species have been shown to exhibit HVA in response to fluctuations in habitat temperature. Williams and Somero (1996) demonstrated that the intertidal mussel, Mytilus californianus, remodels gill phospholipids during different thermal environments, Dahlhoff and Somero (1993) found a strong correlation among mitochondrial membrane fluidity, intertidal zone distribution, and habitat temperature. Seasonal changes in membrane lipids have been found in blue mussel, Mytilus edulis (Pernet et al. 2008), the subtidal eastern oyster, Crassostrea virginica (Pernet et al. 2008), and the hard clam, Mercenaria mercenaria (Parent et al. 2008). While these species may experience significant thermal fluctuations (Pernet et al. 2008), gastropod mollusks that live in the highest regions of the intertidal zone (e.g. Littorina sp.) generally experience much greater thermal fluctuations (Garrity 1984, McQuaid and Scherman 1988; Cleland and McMahon 1990; Jones and Boulding 1999) and therefore face a greater challenge to membrane integrity in the face of more extreme thermal variation.

To determine whether gastropod mollusks modify their membrane order during thermal excursions commonly experienced in the intertidal zone, we examined membrane fluidity in a selection of marine intertidal gastropods under three scenarios: (1) laboratory thermal acclimation, (2) short-term acclimatization during extreme thermal conditions in the natural environment when midday low tides coincide with hot weather (Helmuth et al. 2002), and (3) acclimatization to a vertical range of thermal microhabitats spanning the marine intertidal zone. Under scenario one, we examined membrane fluidity in the mid-intertidal snail Tegula funebralis acclimated to a 20°C range of temperature. In scenario two, we examined membrane fluidities of Littorina keenae individuals sampled from two intertidal zone microhabitats at the beginning and end of a hot midday low tide; one microhabitat was protected from waves and wind and was hotter than the other microhabitat that was exposed to waves and wind. In both microhabitats L. keenae were situated in the upper regions of the intertidal zone, about three to four feet above the mean lower low water level. For the third scenario, we compared membrane fluidities of multiple species of gastropod mollusks from throughout the vertical range of the intertidal zone at Cape Arago, OR.

Materials and methods

Specimen collection and maintenance

Tegula funebralis Specimens were collected from Hopkins Marine Station (36°37’ N; 121°54’ W) on August 21, 2002, and N = 20 individuals were placed into each of three constant-temperature re-circulating aquaria held at 5, 15, or 25°C (±1°C). Acclimation temperatures for T. funebralis were within temperature ranges experienced in the natural environment, where body temperatures for this species often change 20–25°C within a 24-h period (Tomanek and Somero 1999, 2000). Snails were fed algae (Macrocystis pyrifera fronds) once per week during acclimation. After 4 weeks of acclimation, snails were frozen on dry ice and stored at −80°C until sample preparation.

Littorina keenae Specimens were collected from exposed and protected sites at Hopkins Marine Station on May 30, 2002, once just before sunrise at 05:30 as the high tide was ebbing, and again at 13:15 near the end of the low tide. Body temperatures (T_b) of snails were obtained by inserting a K-type hypodermic needle thermocouple (Omega) into the foot of each organism. Specimens were then immediately frozen on dry ice and stored each at −80°C until sample preparation.

Cape Arago species Snails from the south cove intertidal zone of Cape Arago, OR (43°21’ N; 124°19’ W) were collected on July 23, 2002, during morning low tide. Snails.
were dissected in the field, and tissue samples were immediately frozen in a liquid nitrogen dry shipper (Taylor Wharton). Samples were transported to Hopkins Marine Station and stored at −80°C until sample preparation.

Sample preparation

Approximately 6 mg gill and mantle tissue from each snail were homogenized in 3.5 ml of iced 50 mmol l⁻¹ phosphate buffer (buffer A) using a Kontes-duall ground glass homogenizer. The small size of some species (especially *L. keenae*, max length = 25 mm) required use of combined gill and mantle tissue to obtain sufficient tissue mass, and for consistency, all samples were prepared in this manner. The 3.5 ml volume of homogenization buffer ensured sufficient volume for analysis. Homogenates were centrifuged at 80 g for 3 min at 4°C, and resulting supernatants were decanted to fresh tubes.

For crude preparations, the supernatants were diluted with buffer A to an absorbance of 0.15 at 364 nm, and 2 ml of the resulting suspension was placed into a plastic fluorescence cuvette designed specifically for use in fluorescence measurements at the wavelengths used in this study (see, e.g. Williams and Somero 1996). To each cuvette, 2 μl of the fluorescent probe (2 mmol L⁻¹ diphenylhexatriene (DPH) in N,N'-dimethylformamide) was added. The cuvette was sealed with parafilm, and the solution was stirred for 30 min in the dark, allowing diffusion of the DPH molecule into the membranes.

For microsome preparations, the crude supernatant (above) was centrifuged at 20,000 g for 1 h at 4°C to remove mitochondria, lysosomes, and peroxisomes. The resulting supernatant was then centrifuged at 100,000 g for 1 h at 4°C to pellet out plasma membrane and microsomal fractions. The resulting pellet was stored under nitrogen gas at −80°C and resuspended in 3 ml iced buffer A immediately before DPH was added as described earlier.

Fluorescence anisotropy (described below) of crude homogenate was compared with fluorescence anisotropy for microsomal preparations to check for consistency. Anisotropy values for the two preparation methods did not significantly differ, although at cold temperatures (≤10°C) data for crude homogenate samples had a greater variance (Fig. 1). Thus, microsome preparations were used whenever sufficient tissue was available (i.e. for thermally acclimated *T. funebralis* and snail species collected from Cape Arago), and crude homogenate was used for the small *L. keenae* samples.

Fluorescence anisotropy measurements

Cuvettes containing prepared samples were equilibrated to the test temperature for 15 min prior to each measurement. Fluorescence anisotropy of the DPH in each sample was determined as described elsewhere (Williams and Somero 1996) using a Perkin-Elmer MPF-44A fluorescence spectrophotometer with spectral band widths set to 16.7 nm at test temperatures ranging from 10 to 35°C. Excitation was at 364 nm and emission was measured at 430 nm. Anisotropy values were determined multiple times for each sample at each test temperature, and average anisotropy values for each sample at each test temperature were used in subsequent analyses.

Statistical analysis

Fluorescence anisotropy values for each sample were plotted as a function of test temperature, and slopes of best-fit lines were generated using linear regression analysis (SPSS, ver. 14.0). These slopes were then used to calculate anisotropy values at 20°C for each sample, and these values were used for comparisons among groups, using analysis of variance (ANOVA, SPSS, ver. 14.0).

Results

Membrane fluidity in laboratory acclimated *Tegula funebralis*

Thermal acclimation of *Tegula funebralis* did not induce any measurable changes in membrane fluidity. Fluores-
Fluorescence anisotropy of DPH was similar in all three acclimation groups at each test temperature, and there were no significant differences in the temperature sensitivity of DPH fluorescence anisotropy among snails acclimated to different temperatures (Fig. 2). The mean (±1 SD) fluorescence anisotropy calculated at 20°C was 0.194 ± 0.011, 0.190 ± 0.022, and 0.208 ± 0.017 for snails acclimated to 5°, 15°, and 25°C, respectively. Acclimation temperature and membrane anisotropy at 20°C showed no significant correlation (ANOVA, \( F_{1,13} = 1.565, \quad P = 0.233, \quad r^2 = 0.107 \), Fig. 3).

Membrane fluidity in field-acclimated Littorina keenae across a tidal cycle

Body temperatures for Littorina keenae fluctuated with tide and time of day at both exposed and protected sites, but snails at the protected site were found to experience more extreme temperature ranges than those at the exposed site. Body temperatures (\( T_b \)) recorded during spring and summer 2002 at the exposed site ranged from 9 to 19°C, whereas measurements at the protected site exhibited a wider fluctuation ranging from 10 to 37°C. On May 30, 2002, a very calm hot day with midday low tide, \( T_b \) values of L. keenae at exposed and protected sites were similar at 05:30 (13.0 ± 0.4°C and 13.1 ± 0.2°C, respectively), but by 13:15, \( T_b \) at exposed and protected sites differed greatly (17.8 ± 1.2°C and 27.5 ± 0.8°C, respectively). On an extremely hot day during an afternoon low tide (July 1, 2002, at 15:00), \( T_b \) of L. keenae at the protected site reached as high as 37.3°C, with a mean \( T_b \) of 35.2 ± 1.8°C.

The large \( T_b \) changes of Littorina keenae during low tide periods were not accompanied by changes in membrane fluidity. Fluorescence anisotropy values did not differ between collection times at either site nor did anisotropy values differ appreciably among snails from exposed and protected sites at any time of collection (Figs. 4, 5). Snails from the protected site had slightly less fluid membranes than snails from the exposed site at the beginning of the low tide in the morning (Fig. 5), and there was a statistically significant effect of site in a multiple linear regression fit (ANOVA \( P < 0.004 \)). However, sampling time had no effect within each site nor did snail membrane fluidities differ significantly between sites in the afternoon.

Membrane fluidity in field-acclimatized gastropods across vertical intertidal zones

In a broad comparison of membrane fluidities in snails from throughout the intertidal zone at Cape Arago, OR, we observed large interspecific variation in fluorescence anisotropy values (Fig. 6), but the differences in membrane fluidity between species were not consistently related to thermal microhabitat (i.e. vertical zonation) (Fig. 7). Anisotropy values for the snails in this study (Fig. 6) had as great a range as those published from fish at body temperatures ranging from 1 to 24°C (Logue et al. 2000). Interspecific variation in mean fluorescence anisotropy values at
20°C was greatest for species from the low intertidal zone, intermediate for species living in the mid-intertidal zone and lowest for high intertidal zone species (Fig. 7).

Discussion

The fluidity of biological membranes tends to be preserved within a narrow range across many species adapted to a
wide range of body temperatures, a phenomenon termed homeoviscous adaptation (HVA) (Sinensky 1974; Cossins and Prosser 1978; Logue et al. 2000). Relatively few studies have examined how membrane fluidity is preserved during the large and rapid temperature changes commonly experienced by intertidal organisms (Dahlhoff and Somero 1993; Williams and Somero 1996). This study aimed at assessing whether intertidal gastropod mollusks reorder their membranes during low-tide thermal excursions, laboratory thermal acclimation, or acclimatization to vertical position in the intertidal zone.

Eurythermal organisms that routinely face such rapid and significant ambient thermal excursions as those in the intertidal zone have been shown to maintain membrane fluidity within the narrow range required for biological function via HVA (Hazel and Williams 1990; Behan-Martin et al. 1993). Body temperatures of snails can increase by over 20°C during summertime low tide periods (this study, Garrity 1984; Tomanek and Somero 1999); thus through direct thermal perturbations, we would expect membrane fluidity to increase dramatically during low tides unless the snails have mechanisms to rapidly and selectively alter and stabilize the fluidity of their membranes. Such observations have been made for the mid-intertidal mussel, *Mytilus californianus*, which has been shown to reorder gill membranes to maintain relatively constant membrane fluidities during seasonal acclimatization and during simulations of the rapid thermal changes associated with low tide (Williams and Somero 1996). Dahlhoff and Somero (1993) showed that fluidity of mitochondrial membranes from species of abalone living across a range of thermal habitats varies with acclimation temperature and thermal habitat. Other bivalves (e.g. oysters and clams) also seasonally vary both phospholipid composition and sterol content of gill tissue in response to simulated seasonal temperature changes (Pernet et al. 2008; Parent et al. 2008). Our data suggest intertidal snails do not alter membrane fluidity during thermal acclimation (Figs. 2, 3) or thermal acclimatization (Figs. 4, 5), and further, interspecific differences in membrane fluidity do not correlate with vertical zonation (Figs. 6, 7). The absence of HVA in intertidal snails is unexpected and raises questions as to what other adaptive strategies may stabilize their membranes at high temperatures.

Homeoviscous adaptation through changes in membrane composition is not the only way an organism can maintain membrane fluidity during thermal perturbations. Membrane fluidity can also be influenced by the milieu surrounding the membrane (Tocanne and Teissié 1990; Hazel et al. 1992). Due to our method of membrane preparation, the fluidity of snail membranes measured in our study should reflect only membrane composition, not differences in composition of the surrounding milieu. Thus, while our results indicate that intertidal marine snails do not make compensatory adjustments to membrane composition in response to thermal acclimation or acclimatization, it is entirely possible that their membranes are stabilized by changes in the surrounding milieu during ambient thermal fluctuations.

Two plausible explanations for how membrane fluidity is maintained over a wide range of temperatures arise as extensions of a “stabilizing milieu” hypothesis. First, high temperature could lower the pH of the milieu surrounding these membranes, causing ionization of phosphate head groups and greater membrane stability without changing membrane composition. During high-temperature excursions, the pH of internal fluids in these snails would likely drop, changing the ionization state of membrane phospholipids and thereby affecting their molecular packing (Tocanne and Teissié 1990). In poikilotherms, intracellular pH varies inversely with body temperature (see, e.g. Hazel et al. 1992). Such decreases in pH could diminish the strength of the charge on phosphate head groups, decreasing their hydration state and causing them to become more densely packed. This condensation of membrane phospholipids would serve to reduce fluidity, thereby stabilizing membranes at high temperatures (Hazel et al. 1992). Indeed, a decrease in pH of the milieu surrounding biological membranes has been shown to reduce membrane fluidity in a variety of organisms (see Hazel et al. 1992 and references therein).

Second, marine gastropods possess high levels of organic osmolytes (e.g. betaine, taurine, and trehalose) (Rosenberg et al. 2006, Tsvetkova and Quinn 1994) that stabilize membrane lipids and some membrane-associated proteins (Rudolph et al. 1986, Tsvetkova and Quinn 1994, Yancey 2005), and in the milieu surrounding membranes may counteract thermal perturbations to membrane fluidity. These organic osmolytes can increase molecular spacing of membrane phospholipids, preventing spontaneous phase transitions at low temperatures. At high temperatures, however, the presence of these osmolytes increases membrane fluidity and energetically favors Hex II phase formation (Tsvetkova and Quinn 1994), which could destabilize membranes (see e.g. Hochachka and Somero 2002). For intertidal zone snails, membranes may be stabilized by reduced pH during periods of high temperature, and potentially organic osmolytes could aid in maintenance of membrane fluidity during periods of low temperature. Organic osmolytes play additional roles in maintaining membrane order during exposure to non-thermal stresses associated with intertidal zone environments, including desiccation (Rudolph et al. 1986; Yancey 2005) and hypoxia (Yancey 2005), but the role of organic osmolytes in thermal acclimation and acclimatization of intertidal zone gastropods remains to be fully understood.

The small size of many specimens we studied necessitated using a combination of gill and mantle tissues in our...
preparations; it is possible that the two tissues respond differently to temperature variations, such that lack of HVA in one tissue masked HVA in the other. Not all tissues exhibit HVA, and in some cases, different tissues within a single organism exhibit different propensities for HVA. Cossins et al. (1978) found no evidence for HVA in sarcoplasmic reticulum (SR), and Crocket and Hazel (1995) found that while basolateral membranes from trout intestinal epithelium showed HVA, brush border membranes did not. In tuna, Fudge et al. (1998) found no evidence for HVA when comparing opposite ends of the visceral rete mirabile, despite an estimated 10°C thermal gradient.

One explanation for tissue-specific membrane responses, offered by Cossins et al. (1978), suggests that if membrane function is not influenced by changes in membrane composition (i.e. viscotropic effects), HVA may not be beneficial. They surveyed SR membrane fluidity from a range of species and found no evidence of HVA, despite the fact previous and subsequent studies have shown synaptic membranes from many of the same species exhibit near perfect HVA (Cossins and Prosser 1978; Behan-Martin et al. 1993). Cossins et al. (1978) speculated that if the primary function of the SR (i.e. Ca²⁺-stimulated ATPase activity) is not influenced by viscotropic effects (as indicated by previous studies, see Cossins et al. 1978), then HVA would serve no purpose in this membrane.

It is also possible that if membranes experience hostile conditions in the microenvironment, HVA may not be favorable. Crockett and Hazel (1995) demonstrated that basolateral membranes from trout intestinal epithelial cells varied acyl-chain composition with temperature but that the brush border membranes did not, and in fact, increased fluidity with increasing temperature. They attributed this lack of HVA in brush border membranes to the presence of bile in the apical microenvironment, which might impose unusual requirements on membrane order. Similarly, Pernet et al. (2008) found that gill and digestive gland membranes from both Mytilus edulis and Crassostrea virginica differed in their responses to temperature. Gill membranes showed a near perfect increase in fluidity with decreasing temperature, whereas fluidity of digestive gland membranes varied across a much narrower temperature range. Pernet et al. (2008) attributed the different response of digestive gland membranes to the proximity of digestive enzymes (e.g. lipases) and acids, regular exposure to which might suggest a need for greater membrane stability.

In our study, if lack of HVA in one tissue concealed HVA in the other, there would be two possibilities. First, mantle tissue exhibits HVA, but this fact was masked by the lack of HVA in gill membranes. Second, gill membranes exhibit HVA, but this observation was concealed by the lack of HVA in mantle membranes. While either result is possible, we suggest both are unlikely. First, gill membranes have been repeatedly shown to exhibit near perfect HVA (Williams and Somero 1996; Pernet et al. 2008; Parent et al. 2008). Thus, we suspect that if these species were to exhibit HVA, it would be observed in gills, making it unlikely that lack of HVA in gill membranes masked HVA in mantle membranes. Second, mantle tissue is not typically exposed to such inhospitable conditions as those involving digestive enzymes or acids, and its roles in shell formation (Chave 1984) and osmo- and ionic-regulation (Little 1981) would likely be vulnerable to viscotropic effects and membrane instability. We therefore see no reason to assume gill and mantle membranes should differ in their propensity to exhibit HVA, and thus it seems unlikely that lack of HVA in mantle tissue obscured evidence of HVA in gill membranes.

The remaining, if unexpected, alternative is that neither gill nor mantle membranes of the tested intertidal gastropods exhibit HVA, and as previously described, changes in the surrounding milieu may help maintain proper membrane fluidity. Experiments involving larger gastropod species, in which gill and mantle tissues could be analyzed separately, would be valuable in testing our current hypotheses. Moreover, characterization of the internal aqueous microenvironment of marine gastropod tissues during thermal acclimation or acclimatization could account for the lack of HVA observed in our study and clarify whether marine gastropods have evolved different mechanisms to cope with thermal fluctuations.

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References

Behan-Martin MJ, Jones GR, Bowler K, Cossins AR (1993) A near perfect temperature adaptation of bilayer order in vertebrate brain membranes. Biochim Biophys Acta 1151:216–222
Chave KE (1984) Physics and chemistry of biomineralization. Ann Rev Earth Planet Sci 12:293–305
Cleland JD, McMahon RF (1990) Upper thermal limit of nine intertidal gastropod species from a Hong Kong rocky shore in relation to vertical distribution and desiccation associated with evaporative cooling. In: Morton B (ed) The marine flora and fauna of Hong Kong and Southern China II, vol 3. Behaviour, Hong Kong University Press, Hong Kong, pp 1141–1152
Cossins AR (1983) The adjustment of membrane fluidity during thermal adaptation. In: Cossins AR, Sheterline P (eds) Cellular acclimatization to environmental change. Cambridge University Press, Hong Kong and Southern China II, pp 1141–1152
Cossins AR (1983) The adjustment of membrane fluidity during thermal adaptation. In: Cossins AR, Sheterline P (eds) Cellular acclimatization to environmental change. Cambridge University Press, Hong Kong and Southern China II, pp 1141–1152
Cossins AR, Prosser CL (1978) Evolutionary adaptation of membranes to temperature. Proc Nat Acad Sci USA 75:2040–2043
Cossins AR, Christiansen J, Prosser CL (1978) Adaptation of biological membranes to temperature: lack of homeoviscous adaptation
