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SEASONAL-, TIDAL-CYCLE- AND MICROHABITAT-RELATED VARIATION IN MEMBRANE ORDER OF PHOSPHOLIPID VESICLES FROM GILLS OF THE INTERTIDAL MUSSEL *MYTILUS CALIFORNIANUS*

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Summary

Alterations in the order, or fluidity, of cellular membranes in response to variations in environmental temperature are well known. The mussel *Mytilus californianus*, a common inhabitant of mid-intertidal regions along the Pacific coast of North America, can experience large (20 °C or more) and cyclic (every 6 h) changes in body temperature (T_b) during tidal cycles. In the present study, we explore membrane order during seasonal and tidal temperature cycles and find that vesicles prepared from gill phospholipids exhibit significant seasonal differences in order that are consistent with homeoviscous adaptation and suggest winter-acclimatization to mean T_b values and summer-acclimatization to upper extreme T_b values or to large cyclic thermal fluctuations, despite repeated resubmergence in sea water at 10 °C during both seasons. Phospholipid vesicles prepared from the gills of mussels acclimated for 6 weeks to constant high or low temperatures in the laboratory fail to exhibit temperature-compensatory differences in order. In addition, during the summer, mussels inhabiting high intertidal sites, but not those from low sites, possess the ability to alter membrane order rapidly (within hours). This alteration of order appears to represent a mechanism designed to offset the thermal variations encountered during the tidal cycle. Thus, *M. californianus* have the ability to adjust membrane order on seasonal as well as hourly time scales and do so on the basis of their height in the intertidal region.

Key words: membrane order, membrane fluidity, homeoviscous adaptation, fluorescence polarization, *Mytilus californianus*.

Introduction

Many critical cellular activities are associated with biological membranes (Yeagle, 1992). Because these structures consist of ordered aggregates of weakly interacting molecules (i.e. hydrophobic and other non-covalent interactions among phospholipids and proteins), their normal functioning is susceptible to disruption by changes in temperature (Hazel and Williams, 1990; Hazel, 1995). A decrease in temperature causes an immediate increase in the overall packing order of membrane phospholipids (commonly referred to as a decrease in membrane fluidity) and elevated temperature decreases membrane order. Large changes in order, in either direction, can lead to membrane dysfunction, but even small changes can significantly influence membrane-associated processes (Hazel and Williams, 1990). Poikilotherms inhabiting environments characterized by changing temperatures are able to counteract this disrupting effect of temperature. One way they do so is by altering the phospholipid packing order of their cellular membranes by modifying phospholipid composition or membrane structure. Thus, after exposure of conspecifics to different environmental temperatures for a sufficient period, the order of their membranes, when measured at a common temperature, is usually different, with membranes from warm-acclimated individuals considerably more ordered than corresponding membranes from cold-acclimated individuals. When measured at the respective acclimation temperatures, however, the orders of the membranes are often found to be equal. This defense of a particular membrane order in spite of altered environmental temperature has been termed homeoviscous adaptation (HVA; Sinensky, 1974; Behan-Martin *et al.* 1993).

Alterations in membrane order consistent with HVA are well documented in animals exposed to different temperatures for relatively long periods (weeks to months) (Hazel and Williams, 1990), but the time course of this process and its role in

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acclimation to rapid temperature changes has only recently begun to be explored. Some organisms routinely encounter rapidly changing temperatures in their environment; for example, at power station effluents (Widdows, 1976), in desert waters (Brown and Feldmeth, 1971; Carey and Hazel, 1989) and in short-term, cyclically changing temperature regimes such as those experienced by vertical migrators (McLaren, 1963) and inhabitants of intertidal regions (Newell, 1976; Hofmann and Somero, 1995). Recent evidence indicates that the onset of homeoviscous adjustments can occur on a time scale faster than previously appreciated. The order of various fish cell membranes, for instance, can respond adaptively to changes in temperature on a time scale of a few to ten hours (Wodtke and Cossins, 1991; Williams and Hazel, 1994) and can be complete and fully reversible in minutes (Dey and Farkas, 1992). The homeoviscous responses of animals experiencing cyclic fluctuations in temperature remain largely unexplored. At present, it is unknown whether organisms experiencing cyclic changes in temperature continuously alter membrane order, and pay the obligatory metabolic price, or allow membrane order to vary and endure the physiological consequences of such variability. If the latter occurs, to which temperature does the organism adjust, the highest, the lowest or the average? This question has seldomly been addressed in studies of temperature adaptation.

The temperate rocky intertidal habitat common along the Pacific northwestern United States has many qualities that make it an exceptional site for investigating the concepts mentioned above. The prevailing ocean currents maintain seawater temperatures of approximately 10 °C year-round (Elvin and Gonor, 1979). Regardless of season, seawater temperature rarely drops below 8 °C or exceeds 14 °C. Furthermore, the central coast of Oregon is under the influence of a mixed semidiurnal tidal flux and is also characterized by very unpredictable and highly variable terrestrial temperatures. These factors combine to ensure that, throughout the year, the local intertidal inhabitants experience a relatively invariant thermal environment (sea water) every 6 h, but then must face the prevailing terrestrial thermal regime during the following 6 h, a regime that can include hot summer days and cold winter nights. By almost any standard, this can be considered a challenging thermal environment.

The California mussel *Mytilus californianus* is common along the west coast of North America, and musselbeds dominate wide vertical sections of the mid intertidal zone in many locations (Morris *et al.* 1980). Thus, conspecifics experience very different thermal regimes, even at the same rocky intertidal site. Individuals close to the littoral zone (i.e. low sites) are uncovered only briefly during low tide, but individuals near the upper end of the distribution (i.e. high sites) are uncovered for several hours between high tides. Body temperatures of mussels of the genus *Mytilus* may exceed 30 °C during mid-day low tides (Elvin and Gonor, 1979; Hofmann and Somero, 1995). Accordingly, there are significant differences in the physiology of mussels and other invertebrates inhabiting high *versus* low intertidal sites. They exhibit, for example, differences in thermal stability of enzymes and mitochondrial function (Dahlhoff and Somero, 1993*a*,*b*), concentrations of heat shock proteins and ubiquinated proteins (Hofmann and Somero, 1995), metabolic rate (Markel, 1974; Anderson, 1978; Wilson and Elkaim, 1991), heart rate (Segal *et al.* 1953; Segal, 1956; Pickens, 1965; Markel, 1974), mean lethal temperature (Tyler-Walters and Davenport, 1990), survival time at elevated temperature (Anderson, 1978) and resistance to desiccation (Kensler, 1967). Thus, there is ample reason to suspect that differences might exist in adaptation of cellular membrane order. In this work, we examine seasonal, tidal and microhabitat-dependent (intertidal height) variation in the membrane order of phospholipid vesicles from the gills of *Mytilus californianus*. We find that these organisms display considerable plasticity in membrane order over all of these temporal and spatial scales.

Materials and methods

Materials

N-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) (Hepes) and silicic acid (mesh size 60–200) were purchased from Sigma Chemical Co. (St Louis, MO, USA); 6-diphenyl-1,3,5-hexatriene (DPH) was from Molecular Probes (Eugene, OR, USA). All solvents used were analytical reagent grade or better.

Study site and collection of in situ *body temperatures*

The study site, Strawberry Hill (44°15′ N, 124° 07′ W), near Cape Perpetua, Oregon, USA, is a basaltic outcropping with very complex topography including numerous pools, channels and open benches. The semidiurnal tidal range is 2.5 m. Specimens of the mussel *Mytilus californianus* (Conrad) were sampled from high [+2.1 m above mean low water (AMLW)], middle (+1.3 m AMLW) and low (+0.6 m AMLW) sites from within the mid intertidal range of the mussels. The community structure of Strawberry Hill, including the distribution of *M. californianus*, has been well described (Menge, 1992; Menge *et al.* 1994).

Mussel body temperatures (T_b values) were measured *in situ* using a hand-held electronic thermometer (model HH82, Omega Engineering, Stamford, CT, USA) fitted with small, K-type thermocouples. The thermocouple probes were inserted into the mussel body cavity through a small hole (2.5 mm diameter) that had been drilled into the anterior region of the shell. The probes were held in place by small pieces of modeling clay that also prevented excessive water loss.

Tissue collection from field-acclimatized and laboratory-acclimated mussels

Mussel gill tissue was collected at the field site in summer (August) and winter (February) from animals inhabiting the high and low intertidal sites (=field-acclimatized mussels). For the assessment of seasonal effects, tissue was collected from animals at both sites during low tide, but without specific regard to time of emersion, i.e. specimens included animals

gathered near the beginning, as well as near the end, of emersion. For the microhabitat analyses, gills were collected at both sites from animals minutes after the tide had receded (=post-emersion) and again just prior to submergence by the returning tide (=pre-immersion). Depending on tidal height, wind and other factors, high-site mussels are exposed to air for up to 6 h and low-site mussels are exposed for up to 3 h during every 12 h portion of the tidal cycle. Gills were excised with scissors and quickly frozen in foil over an aluminum block cooled with solid CO_2. Tissue samples were held on solid CO_2 before being brought back to the laboratory, where they were stored at −80 °C until lipid extraction.

Mussels were laboratory-acclimated to 10 °C to simulate winter conditions (see below) and to 20 °C, the highest temperature at which these mussels can survive for extended periods (E. E. Williams and G. N. Somero, personal observations; see Almada-Villela *et al.* 1982). Mussels were collected at the middle intertidal site in summer and winter and subsequently held (continuously submerged) for 12 h in 75 l recirculating, refrigerated aquaria maintained at 10 °C. For both seasonal collections, half of the mussels were subsequently maintained at 10 °C while the temperature of the other half was slowly raised to 20 °C over 14 days. All animals were fed dissolved trout pellets twice weekly and held at the final acclimation temperature for 6 weeks. The aquaria were exposed to the natural photoperiod. All mussels treated in this way appeared healthy at the end of the acclimation period, e.g. they rapidly closed their valves when disturbed; none died during the acclimation period. Following acclimation, gill tissue was excised, frozen and stored as described above.

Tide simulation

Owing to the impracticality of collecting mussels from the Strawberry Hill site at high tide, tidal flux was simulated in the laboratory by sequentially exposing freshly collected (at low tide) mussels to room air under bare-bulb illumination (60 W, elevated 10 cm; =low tide), and to water in aquaria thermostatted at 10 °C (=high tide). Emersion and immersion times duplicated those experienced by mussels in the field. The T_b values of two individuals were continuously monitored using the electronic thermometer described above with the thermocouples inserted between the valves. At each sampling interval, gill tissue was dissected, quick-frozen and stored as described above.

Lipid extraction

After weighing, frozen tissue was finely minced in a small volume of $CH_3OH/CHCl_3$ (2:1) containing 0.01 % butylated hydroxytoluene as an antioxidant. Total tissue lipids were extracted (Bligh and Dyer, 1959) and concentrated prior to the separation of neutral lipids from phospholipids by adsorption chromatography on silicic acid (Wren, 1960). A portion of the total lipid extract was applied to the column (<1 mg g^{-1} adsorbent) and eluted with 10 column volumes of $CHCl_3$ followed by 10 volumes of CH_3OH. After elution, the purified phospholipids were concentrated in a rotary evaporator, gassed with N_2 and stored at −20 °C until used for vesicle formation.

Vesicle formation and fluorescence polarization measurements

Stable, plurilamellar vesicles (SPLVs) were chosen for fluorescence polarization analyses because of their stability, low osmotic stress and capacity to mimic biological membranes (Grunner *et al.* 1985). SPLVs were prepared by adding 5.0 ml of water-saturated diethyl ether to a dry film of 2.5 mg of tissue phospholipid previously prepared by rotary evaporation in a round-bottomed flask. After the addition of 0.3 ml of vesicle buffer (72.5 mmol l^{-1} KCl, 72.5 mmol l^{-1} NaCl, 10 mmol l^{-1} Hepes, pH 7.4), the ether was removed under a gentle stream of N_2 while the mixture was sonicated at 40 °C in a bath-type sonicator (80 W, Bransonic 12). The resulting paste was resuspended in 2 ml of vesicle buffer by vortexing, and the vesicles were allowed to equilibrate for 2 h while shaking at room temperature. After equilibration, 3 ml of vesicle buffer was added and the vesicles were collected by centrifugation at 10 400 $\boldsymbol{g}_{av}$ (10 000 revs min^{-1}, Sorvall SS-34 rotor) for 10 min.

Membrane order was assessed by diluting SPLVs with vesicle buffer to an absorbance of less than 0.15 absorbance units at 364 nm before 3 µl of 2 mmol l^{-1} DPH in *N*′,*N*′-dimethylformamide was added to each 4 ml of vesicle suspension. DPH incorporation into the vesicles was allowed to proceed for 30 min while the mixture was slowly stirred in the dark. Polarization of fluorescence emitted by DPH, directly related to membrane order, was calculated and corrected for monochromater polarization (G-factor) using equations described elsewhere (Litman and Barenholz, 1982). A Perkin-Elmer MPF-44A fluorescence spectrophotometer with spectral band widths set to 16.7 nm was used for the measurements, which were carried out over the range 5–40 °C. Excitation was at 364 nm and emission was measured at 430 nm. Vesicle preparations were allowed to stand for 15 min at each assay temperature before readings were initiated, and individual preparations were measured in duplicate at each assay temperature.

Statistics

Comparisons of means were accomplished using Student's *t*-test. Multiple comparisons were performed using one-way, two-tailed analysis of variance (ANOVA). In all cases, *P* values lower than 0.05 were considered significant.

Results

Mytilus californianus populating Strawberry Hill, Oregon, USA, are subject to relatively large, rapid and cyclic changes in T_b. Fig. 1 shows representative T_b values of nine individual mussels on 7 July 1993 (low water −0.21 m at 09:15 h). Although air temperature rose only a few degrees (Fig. 1A), the T_b values of mussels at both the high (Fig. 1A) and low (Fig. 1B) sites increased by as much as 15–18 °C during the

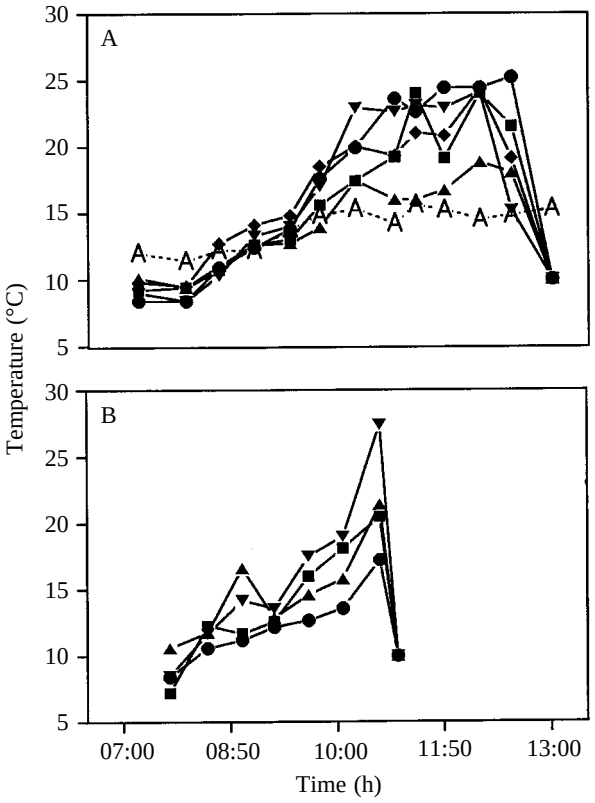

Fig. 1. Body temperatures of nine individual mussels as a function of time during tidal emersion on a clear summer day (7 July 1993). Low tide occurred at 09:15 h. (A) Mussels inhabiting the high (+2.1 m AMLW) intertidal site. The dashed curve with the A symbol represents air temperature. (B) Mussels inhabiting the low (+0.6 m AMLW) intertidal site.

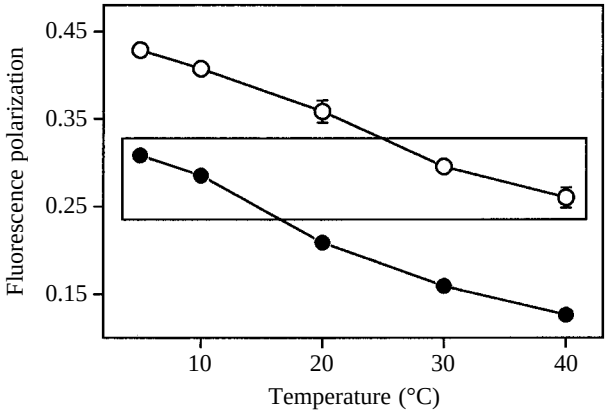

Fig. 2. Fluorescence polarization (the inverse of membrane order) of DPH incorporated into SPLVs formed from phospholipids extracted from the gills of summer- (open symbols, N=12) and winter- (filled symbols, N=20) acclimatized *Mytilus californianus* as a function of assay temperature. The rectangle encloses polarization values common to the two curves. At every temperature, the values are highly significantly different (Student's t-test, P<0.001). Error bars (S.E.M.) are in most cases smaller than the symbols.

period of emersion. Mussels at the high site remained at elevated temperature for nearly twice as long as individuals from the low site. At both sites, T_b values dropped rapidly as the tide returned and covered the mussels.

Within both the laboratory-acclimated and field-acclimatized groups, no significant differences in total gill lipid or total gill phospholipid content were detected. When individuals from the laboratory and field groups were compared with each other, however, it was found that gills from mussels held in the laboratory contained a significantly lower total lipid (12.6±1.5 mg g^{-1} gill, mean ± S.E.M., N=16 *versus* 28.4±3.0 mg g^{-1} gill, N=31) and phospholipid (2.78±0.30 mg g^{-1} gill, N=16 *versus* 8.41±1.1 mg g^{-1} gill, N=30) content than gills from the field-acclimatized animals. The neutral lipid contents of field and laboratory groups were not significantly different even though the field-acclimatized group showed a significantly higher neutral lipid content in gills from winter-acclimatized mussels (5.855±0.764 mg g^{-1} gill, N=20) compared with summer-acclimatized animals (3.315±0.766 mg g^{-1} gill, N=12).

The polarization of fluorescence emitted by DPH incorporated into SPLVs formed from phospholipids extracted from *M. californianus* gill tissue (collected while the tide was out but without regard to specific emersion status, see Materials and methods) revealed large seasonal differences in membrane order (Fig. 2). At all temperatures tested, vesicles prepared from gills of summer-acclimatized mussels were significantly more ordered (i.e. exhibited higher polarization values) than vesicles from winter-acclimatized mussels. Animals from both seasons had similar membrane order in the region indicated by the rectangle in Fig. 2. If homeoviscous adaptation in these animals is complete (i.e. membranes have similar order when measured at the temperature of acclimatization; see Behan-Martin *et al.* 1993), then the region of equal membrane order corresponds to the temperature to which the animals have adapted the order of their membranes. Thus, the winter mussels appear to be adapted to temperatures between 5 and 15 °C and the summer mussels to temperatures between 25 and 40 °C.

When winter-acclimatized mussels were laboratory-acclimated to 10 or 20 °C for 6 weeks, phospholipid SPLVs prepared from the gills of both groups displayed membrane order that was not significantly different from each other or from that of vesicles from winter-acclimatized mussels sampled in the field (Fig. 3A, compare with Fig. 2). In contrast, when summer-acclimatized mussels were laboratory-acclimated for 6 weeks to 10 or 20 °C, both groups exhibited order values much lower than those found in summer animals sampled in the field (Fig. 3B, compare with Fig. 2). Summer-acclimatized animals after acclimation to 10 °C exhibited membrane order values similar to those of winter-acclimatized animals and to those of winter animals after laboratory acclimation to both 10 and 20 °C. However, when measured between 5 and 20 °C, the membrane order of SPLVs from summer-acclimatized animals acclimated to 20 °C, though reduced when compared with that from SPLVs of summer-

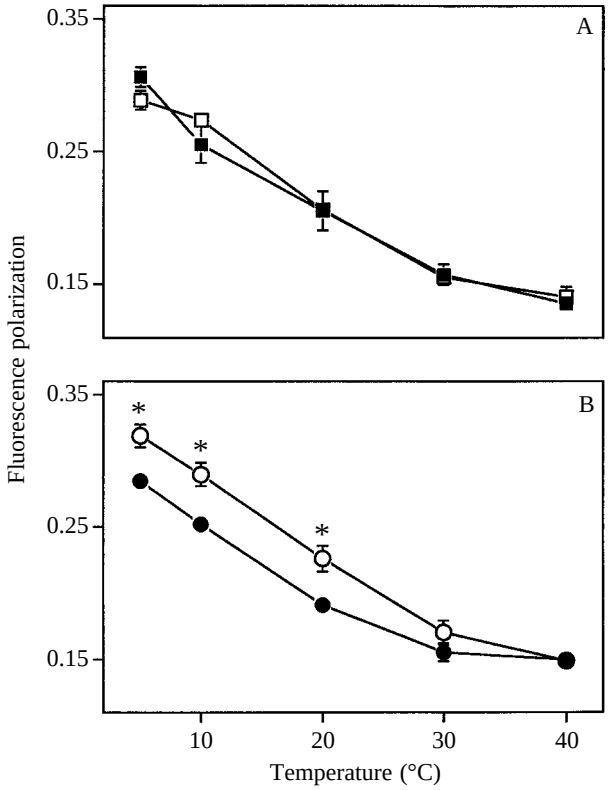

Fig. 3. Fluorescence polarization of DPH incorporated into SPLVs formed from phospholipids extracted from the gills of (A) winter- and (B) summer-acclimatized mussels after 6 weeks of laboratory acclimation to 20 °C (open symbols, N=4, N=3, respectively) or 10 °C (filled symbols, N=4, N=5, respectively) as a function of assay temperature. Asterisks indicate pairs of means that are significantly different (Student's t-test, P<0.05). Values are means ± S.E.M.

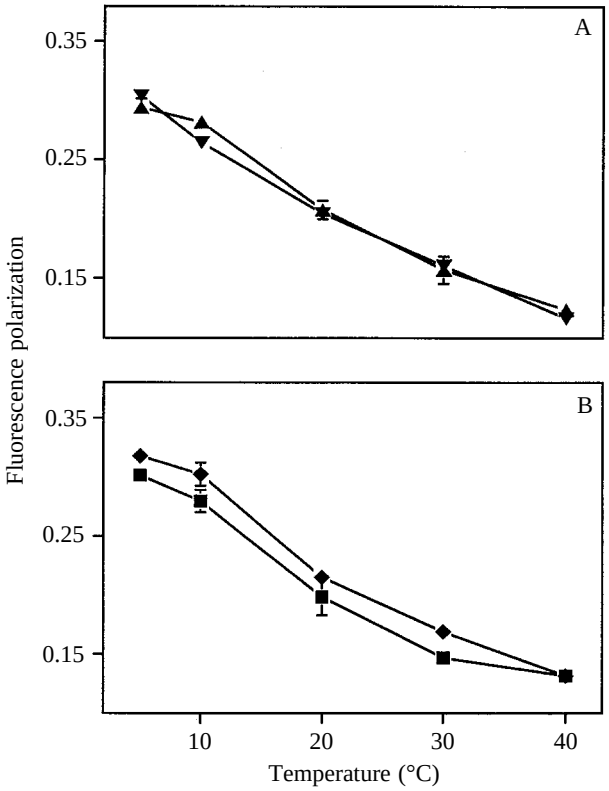

Fig. 4. Fluorescence polarization of DPH incorporated into SPLVs formed from phospholipids extracted from the gills of winter-acclimatized mussels as a function of assay temperature. (A) Vesicles prepared from tissues of high intertidal mussels collected post-emersion (▼, N=5) and pre-immersion (▲, N=5). (B) Vesicles prepared from tissues of low intertidal mussels collected post-emersion (squares, N=5) and pre-immersion (diamonds, N=5). Values are means ± S.E.M.

acclimatized mussels sampled in the field (Fig. 2), was significantly higher than that from summer-acclimatized animals after acclimation to 10 °C (Fig. 3B).

In order to explore the changes in membrane order that might occur in gills during emersion, i.e. over the time course of a single low tide, tissue was collected post-emersion and pre-immersion from individuals from high and low intertidal sites. Fig. 4A reveals that in winter (5 February 1994, low tide=+0.12 m AMLW at 15:00 h, seawater temperature=10.2 °C, maximum recorded T_b=26.5 °C) there were no significant differences in membrane order between SPLVs from post-emersion and pre-immersion mussels inhabiting either the high or the low intertidal sites. There was, however, a consistent though insignificant trend in SPLVs prepared from the gills of low intertidal mussels to exhibit increased membrane order at the end of low tide, i.e. after several hours of aerial exposure (evident at all temperature except 40 °C, Fig. 4B).

In summer too (7 August 1993, low tide=–0.21 m AMLW at 09:15 h, seawater temperature=10.0 °C, maximum recorded T_b=27.4 °C), membrane orders of SPLVs from low intertidal mussels before and after aerial exposure are not significantly different, except at 5 °C, where, in contrast to the low intertidal mussels sampled in winter, vesicles exhibited a significantly lower order after exposure to the terrestrial environment (Fig. 5B). SPLVs prepared from mussels from the high intertidal region, in contrast, showed a significant and consistent difference between post-emersion and pre-immersion periods (Fig. 5A). When measured between 5 and 30 °C, SPLVs made from phospholipids taken from mussels after a period of aerial exposure (and elevated T_b values) were significantly less ordered than those from comparable mussels collected immediately following several hours of immersion in sea water at 10 °C (Fig. 5A).

The significant differences observed in field-collected, summer-acclimatized mussels from the high site during the tidal cycle (Fig. 5A) were examined with increased temporal resolution by collecting gill tissue from summer-acclimatized, high-site mussels at 1.5 h intervals during a simulated tidal cycle. Mussels collected from the field site at the beginning of low tide were transported to the laboratory (a 30 min trip at ambient temperature) and then sequentially exposed to bare-bulb illumination on the bench (to imitate low tide) and to seawater aquaria at 10 °C (to imitate high tide). The extent of each exposure was carefully timed to correspond to the tidal conditions experienced on that day by mussels in the field. The

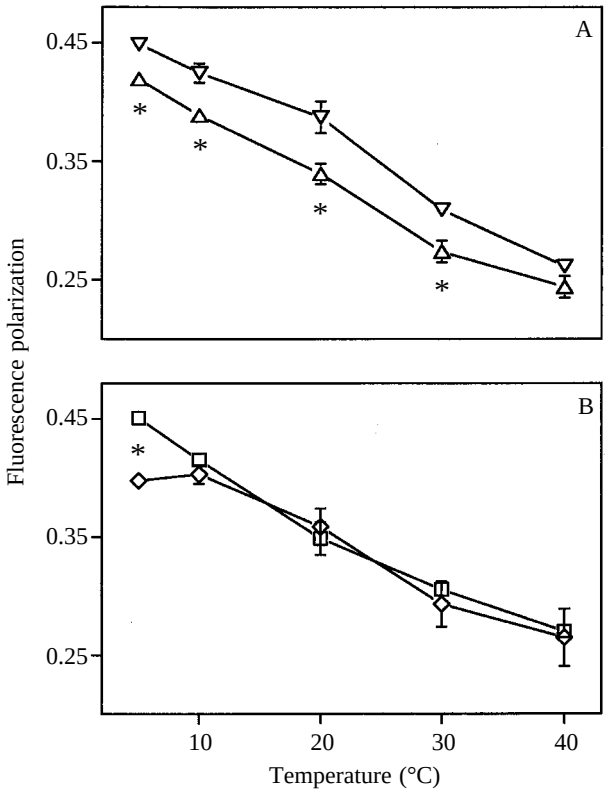

Fig. 5. Fluorescence polarization of DPH incorporated into SPLVs formed from phospholipids extracted from the gills of summer-acclimatized mussels. (A) Vesicles prepared from tissues of high intertidal mussels collected post-emersion (▽, N=3) and pre-immersion (△, N=3). (B) Vesicles prepared from tissues of low intertidal mussels collected post-emersion (squares, N=3) and pre-immersion (diamonds, N=3). Asterisks indicate pairs of means that are significantly different (Student's t-test, P<0.05). Values are means ± S.E.M.

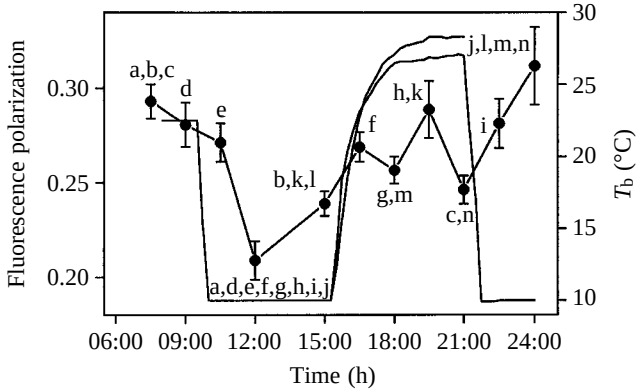

Fig. 6. Changes in membrane order of phospholipid vesicles from gills of summer-acclimatized *Mytilus californianus* subjected to simulated tidal cycles. The temporal variation in the fluorescence polarization of DPH, measured at 20 °C, is shown (circles, each point represents the mean ± S.E.M. of three different individuals). The same pattern was observed at all assay temperatures. Pairs of values that are significantly different (ANOVA, P<0.05) are labeled with the same letter. The T_b values of two individuals are indicated by the lines without symbols (temperature data were terminated in one of these individuals when it was removed and sampled).

data in Fig. 6, which show mussel body temperature and fluorescence polarization of phospholipid SPLVs, confirm that membrane order in *M. californianus* changes significantly within hours. When measured at a constant temperature (20 °C), vesicles formed from gill phospholipids of animals collected after 2.5 h of immersion (at 12:00 h), a time when T_b values were measured to be 10 °C (Fig. 6), were significantly less ordered than vesicles prepared from gills during exposure to air and whose T_b values were 22.5 °C (at 07:30 h and 09:00 h). SPLVs prepared from animals collected near the middle of high tide (12:00 h) also exhibited significantly lower order than vesicles prepared from animals collected after 1 h of immersion (10:30 h). Thus, a significant drop in membrane order was observed in animals collected 1.5 h apart. After 12:00 h, membrane order began to rise. The increase in order appeared before emersion, while mussel T_b values were 10 °C, and continued after emersion until the midpoint of low tide was reached at 19:30 h. By that time, the order of gill phospholipid SPLVs had returned to values observed at the beginning of the experiment and T_b values had reached 26–28 °C. During a subsequent round of simulated high tide beginning at 21:30 h, T_b values again dropped rapidly, but the order of SPLVs obtained from animals collected during this second immersion exhibited values among the highest observed (Fig. 6).

Discussion

Mytilus californianus inhabiting the central Oregon coast routinely experience large and rapid fluctuations in T_b (e.g. Fig. 1). Although the animals depicted in Fig. 1 reached a maximum body temperature of 25–28 °C, animals at this site are known to experience T_b values as high as 34 °C in the summer (Gonor, 1968). Mathematical models based on measured T_b values, tidal fluxes, solar irradiation and other factors suggest that somewhat higher temperatures may be attained by mussels at the high intertidal sites (Elvin and Gonor, 1979). High body temperatures are not restricted to the summer months, however, as mussel T_b values over 30 °C also occur in winter on mild, cloudless days (data not shown). Therefore, it is possible for mussels to experience T_b values up to 30 °C at all times of the year, i.e. 20 °C variation in T_b can occur at all seasons. Neither the regional air nor sea water ever approaches these temperatures; the high T_b values are a result of solar irradiation on these darkly colored mussels. Thus, not only season and tidal height, but also orientation to the sun, contribute to determining T_b values of these mussels.

We found no seasonal variation in the total lipid or phospholipid content of *M. californianus* gill from animals collected in the field. This is consistent with previous studies of gill lipids in the closely related *M. edulis* (Zurburg *et al.* 1979; Zandee *et al.* 1980), and in the total body lipids of *M. galloprovinciallis* (Miletic *et al.* 1991) and other bivalves (Ansell *et al.* 1964; Ansell and Trevallion, 1967; Nevenzel *et*

al. 1985). The seasonal invariability of gill lipid content may be a common feature among the pelecypoda. The phospholipid and neutral lipid content of the gill reported here for *M. californianus* is consistent with previous reports employing similar methods (Zandee *et al.* 1980; Nevenzel *et al.* 1985). The reduced total lipid and phospholipid content of laboratory-acclimated mussels compared with that of animals collected and sampled in the field may reflect differences in the lipid content of the pelleted *versus* natural diets consumed during the study. Though the total gill lipid content of laboratory-acclimated mussels was reduced, the animals appeared healthy and responded to tactile stimuli in a manner similar to that of animals observed in the field.

Even though *M. californianus* at Strawberry Hill experience a seawater temperature of approximately 10 °C every 6 h throughout the year, phospholipid SPLVs prepared from gill tissue of summer- and winter-acclimatized animals nevertheless exhibited a strong seasonal variation in order consistent with homeoviscous adaptation (Fig. 2). At all temperatures measured between 5 and 40 °C, vesicles from summer-acclimatized mussels were significantly more ordered than those from winter animals, reflecting differences in membrane structure or composition presumably allowing retention of membrane function at the elevated (i.e. order-decreasing) temperatures prevalent during the summer (Hazel and Williams, 1990). Because these SPLVs were formed from isolated phospholipids, the differences in order reflect differences in membrane phospholipid composition between summer and winter animals. Differences in other membrane constituents, such as sterols, and the contribution of membrane structural complexities (i.e. membrane hemilayers; Williams and Hazel, 1994*b*; and microdomains) could further affect membrane order.

The fact that biological membranes are widely involved in a diversity of cellular activities and functions suggests that membrane order is regulated. It thus seems sensible to expect that seasonal acclimation should lead to complete homeoviscous adaptation (i.e. to a homeoviscous efficacy of one), but this is not always the case (Hazel and Williams, 1990). Though the value of the efficacy of HVA observed is influenced by the probe used to make the determination, complete HVA has been shown in differently acclimated trout (Behan-Martin *et al.* 1993; Crockett and Hazel, 1995) and in interspecific comparisons of mammals, birds and fishes with widely different adaptation temperatures (Behan-Martin *et al.* 1993), using DPH as the membrane probe. If the seasonal compensation of membrane order in *M. californianus* is complete, then similar values of polarization are expected when each membrane preparation is measured at a temperature characteristic of the habitat of that animal. If, however, HVA is incomplete (homeoviscous efficacy is less than one), then the winter adaptation temperature could be overestimated (but not underestimated) and the summer adaptation temperature underestimated (but not overestimated). Thus, the adaptation temperatures estimated above appear to reflect minimum differences in habitat temperatures. The range of values for membrane order (fluorescence polarization) common to both summer and winter membranes, indicated by the rectangle in Fig. 2, suggests that membrane phospholipids of winter-acclimatized mussels are adapted to temperatures between 5 and 15 °C and that those of summer-acclimatized mussels are adapted to temperatures between approximately 25 and 40 °C. Thus, membranes of *M. californianus* exist with relatively low order during the winter and then increase membrane order in summer to offset the effects of increased temperatures, as has been observed in numerous other species (Hazel and Williams, 1990). Considering the mild nature of the winters in this region of the Pacific northwest, winter body temperatures around 10 °C seem reasonable and reflect the mean environmental temperatures experienced during this season (Gonor, 1968). Summer-acclimatized mussels, in contrast, appear to be adapted to the upper extreme temperatures that may be experienced only briefly during emersion on hot, clear days. Mean body temperatures, calculated either over a complete tidal cycle or during emersion only, would be considerably lower than 25–40 °C. For example, if mussels inhabiting high intertidal sites (and thereby receiving the maximum exposure to sunlight and the terrestrial environment) were to increase T_b at a rate between 2.5 °C h^{-1} (Fig. 1) and 5.0 °C h^{-1} (Gonor, 1968) during a 6 h exposure after emerging from sea water of 10 °C, the T_b would average 12.7–18.8 °C over a 12 h period or up to 17.0–27.5 °C during the 6 h of aerial exposure. These temperatures are similar to those we have measured in the field. Although these animals probably experience T_b values of 30 °C and higher, we have no direct evidence that body temperatures of 40 °C are ever attained. However, the data of Fig. 2 indicate that, as far as membrane order is concerned, such a high temperature could be tolerated and is perhaps anticipated by the organisms. It appears, then, that winter-acclimatized *M. californianus* are adapted to temperatures near the average experienced, while summer-acclimatized mussels are adapted to the extreme upper limits of potential thermal exposure. If summer mussels had indeed experienced a T_b of 40 °C prior to collection, they undoubtedly did so only briefly, indicating that the mussels fashion their membranes to withstand an environmental condition that is experienced for only an exceedingly small percentage of time. In contrast, if the mussels had not encountered 40 °C prior to collection, then the implication is that they anticipate experiencing such a temperature and refashion their membranes to withstand this anticipated temperature extreme. In either case, adaptation to high extremes of temperature probably reflects the fact that, at very low order, biological membranes loose their lamellar structure and become dysfunctional. Indeed, membrane function may be significantly impaired well before the lamellar phase is lost; it is believed that heat death results from the rapid flux of ions at low membrane order (Gladwell *et al.* 1975). Massive heat death of mussels (*M. edulis*) has been reported at high temperatures (Tsuchiya, 1983), so heat stress encountered by mussels in summer can reach lethal levels. The salient and important feature of homeoviscous adaptation in summer-acclimatized mussels appears to be the avoidance of highly

disordered membranes, which may be at the expense of tolerating highly ordered membranes for a considerable fraction of time, i.e. during high tide.

A comparison of the order of SPLVs prepared from mussels field-acclimatizated to cyclic temperatures and laboratory-acclimated to constant temperature suggests a definite gradation of response. Membrane order increased in the sequence: winter-acclimatized animals = winter animals acclimated to 10 °C = winter animals acclimated to 20 °C = summer animals acclimated to 10 °C < summer animals acclimated to 20 °C < summer-acclimatized animals. Considering that summer-acclimatized animals almost certainly experienced the highest temperatures, this sequence is not unexpected. It does, however, underscore the idea that mussels exposed to warm temperatures on a cyclic basis exhibit membranes with the highest order. For instance, phospholipid vesicles prepared from winter-acclimatized animals collected in the field, and from winter-acclimatized animals acclimated to a constant (i.e. non-cycling) temperature of 20 °C, which is near the upper limit of long-term survivability, exhibit nearly identical, low order values (Figs 2, 3). In addition, membrane order is greatly reduced in summer-acclimatized animals after exposure for an extended period to high constant temperature (Figs 2, 3). Thus, it appears that the large increase in membrane order observed in summer-acclimatized mussels is due either to short-term exposure to temperatures above those compatible with long-term survival or to repeated, cyclic exposure to more modest temperatures (or some combination of these factors absent in the laboratory-acclimation experiments). This leads again to the interesting question of whether these mussels anticipate a high temperature and restructure their membranes in advance, or whether they restructure their membranes in response to temperature only after it has been (or is being) experienced. The former would seem to require no more than a seasonal rhythm of membrane restructuring, which laboratory acclimation might disrupt (perhaps because of the absence of a cyclic component). The latter would seem to require a means of rapidly restructuring membranes, on a time scale equal to or less than the interval of emersion.

To address the question of rapid membrane restructuring in *M. californianus*, we measured the post-emersion and pre-immersion membrane order of mussels at both high and low intertidal sites, in winter and summer. The absence of any change in membrane order during emersion in winter-acclimatized high- and low-site mussels (Fig. 4), even though the high-site mussels reached a maximum temperature of 26.5 °C, eliminates aerial exposure itself as a causal factor in differences observed in membrane order and is also consistent with the absence of a response during long-term laboratory acclimation of these mussels to 10 and 20 °C. Either winter-acclimatized mussels are unable to adjust membrane order over a period ranging from 6 h (field) to 6 weeks (laboratory) or the conditions prevailing during the winter and found in the laboratory acclimation experiments are insufficient to warrant such adjustment. However, the clear and significant differences observed in the order of SPLVs prepared from high-site, but not low-site, mussels in the summer seems to establish the capacity of the high-site mussels to alter membrane order within the time span of fractions of an hour. It is interesting that only summer animals of the high site exhibit this short-term response. This observation suggests that some attribute of this site during summer permits or induces (or that winter conditions repress or oppose) the ability to restructure cellular membranes rapidly. It would be interesting to determine whether mussels from the low site have retained this ability and whether there is a gradation in the degree or extent of restructuring between the extremes of the intertidal region. Transplant experiments would establish whether this restructuring capacity is a phenotypic or genotypic adaptation of the high-site mussels. In any case, the direction of the change in membrane order observed in the high-site mussels during emersion, when a maximal T_b of 27.4 °C was experienced, is the opposite of that expected on the basis of homeoviscous theory. A decrease in membrane order after a period of emersion, as seen in Fig. 5A, would tend to augment rather than counter the membrane-disordering effect of the increased temperature experienced during aerial exposure at low tide. However, the decrease in membrane order during emersion would prepare the membranes for the order-increasing effects of low temperature during the next period of immersion. It is becoming increasingly clear, however, that membrane qualities other than order are altered during temperature adaptation (Hazel, 1995), and it is possible that the changes reported in Fig. 5 reflect these alterations.

The relative rapidity and apparently paradoxical shift in membrane order observed at the high intertidal site between post-emersion and pre-immersion mussels in summer (Fig. 5) led to a more temporally resolved tidal flux experiment (Fig. 6). Qualitatively, the paradoxical shift was observed again during the tidal flux experiment (Fig. 6; i.e. the pre-immersion value observed at 21:00 h is lower than the post-emersion value at 16:30 h); however, the difference between the points is not significant. On the basis of the findings discussed below, and because only two time points were sampled in the experiment depicted in Fig. 5, it is difficult to assess the significance and context of the shift to decreased membrane order during emersion (Fig. 5) except, perhaps, to note that the change itself was relatively rapid, large and significant.

The results in Fig. 6 clearly indicate that significant changes in membrane order occur over relatively short intervals; some were accomplished in as little as 1.5 h (e.g. between 10:30 h and 12:00 h). Certain of the observed changes in the order of gill phospholipids appear to be adaptive. For example, when measured at a common temperature, the lowest order value was observed in SPLVs prepared from mussels collected during the middle of high tide, when T_b values were at their lowest (e.g. at 12:00 h). Among the highest order values were those occurring during emersion, when T_b values were at their highest (e.g. at 19:30 h). These data suggest a compensatory role for alterations in membrane order during the tidal cycle.

The ordering effects of cold during high tide were offset by a reduction in membrane order, while the disordering effects of elevated temperature during low tide were offset by an increase in membrane order. This pattern is entirely consistent with homeoviscous theory. However, the finding of a paradoxical shift in membrane order between 21:00 h and 24:00 h in the simulated tidal experiment suggests, as do the the results from the high intertidal mussels shown in Fig. 5, that homeoviscous adaptation to the current temperature is not a consistent feature of the membrane order restructuring process in these organisms. Additional field and laboratory studies are needed to resolve more fully these complex temporal changes and their potential adaptive role. What remains clear is that *M. californianus* is capable of accomplishing rapid changes in membrane order, through changes in membrane phospholipid composition, and uses that ability to alter membrane properties during the tidal cycle.

In summary, we have established that SPLVs prepared from gill phospholipids of summer- and winter-acclimatized *M. californianus* collected in the field possess significantly different membrane order and that this difference is consistent with homeoviscous adaptation to 5–15 °C in the winter and to 25–40 °C in the summer. The data suggest that winter animals are acclimatized to mean T_b while summer animals are acclimatized to maximum T_b, probably to ensure the avoidance of highly disordered membranes during brief exposures to extreme temperatures in summer. We further document, by comparing field-acclimatized and laboratory-acclimated animals, that it is the summer mussels, particularly those from the high intertidal sites, that primarily account for the differences in membrane order. Experiments following the order of phospholipid membranes prepared from mussels post-emersion and pre-immersion, as well as additional experiments following membrane order through a tidal cycle, both indicate that *M. californianus*, like fish (Wodtke and Cossins, 1991; Dey and Farkas, 1992; Williams and Hazel, 1994), are capable of effecting rapid alterations in membrane order, which may be used in counteracting the temperature fluctuations experienced during tidal flux. Thus, *M. californianus* probably use a combination of seasonal and tidal alterations to accomplish membrane restructuring which allows successful adaptation to the thermal environment of the rocky intertidal zone. The specific mechanism(s) of rapid membrane restructuring in *M. californianus* remains to be elucidated, and there is a collection of potential paths to be exploited (Hazel and Williams, 1990). Intertidal molluscs such as *Mytilus californianus* are able to thrive in this challenging thermal environment and, since they are amenable to laboratory manipulation, they may become an organism of choice for continued studies of biochemical adaptation to the environment.

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