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Temperature-Mediated Processes in Teleost Immunity: Homeoviscous Adaptation in Teleost Lymphocytes[1] (41300)

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Abstract. In vitro studies with pinfish (*Lagodon rhomboides*) lymphocytes demonstrated that the temperature to which a fish is acclimated *in vivo* affects the temperature dependence of lymphocyte responses to mitogenic stimulation with conconavalin A (ConA) *in vitro*, i.e., at an *in vitro* temperature of 20°, the lymphocytes of 17° acclimated fish responded to ConA better than the lymphocytes of 27° acclimated fish. Fluidity measurements of plasma membranes from lymphocytes isolated from both cold- and warm-acclimated pinfish were made using fluorescence polarization spectrophotometry and the fluorescent membrane probe, 1,6-diphenyl-1,3,5-hexatriene. While the membranes of cold-acclimated pinfish were more fluid than those of warm-acclimated fish when both were measured at the same temperature, the fluidities were equal when measured at the respective acclimation temperatures. These results support the hypothesis that the process of homeoviscous adaptation of lymphocyte plasma membranes may be an important aspect of teleost immunity.

Environmental temperature has been shown to influence the susceptibility of fish to disease and to markedly affect teleost immune responses. In particular, low temperatures have been found to effectively immunosuppress the antibody response in fish. Although the mechanism of low-temperature immunosuppression in fish has not been elucidated, studies by Avtalion and co-workers (reviewed (1)) utilizing the hapten carrier effect in carp have indicated that the low-temperature-sensitive step may involve the development of carrier specific helper cells. In this context it may be important that *in vitro* studies with lymphocytes from the bluegill have indicated a temperature differential in the mitogenic responses of fish T-like and B-like cells (2). Bluegill T-like cells appeared unable to undergo blastogenic responses at lower temperatures that were still conductive to blastogenesis of B-like cells. In attempting to repeat and extend these *in vitro* findings with another fish species, studies were undertaken with the marine pinfish, *Lagadon rhomboides*. Although the initial results with pinfish lymphocytes failed to demonstrate significant *in vitro* responses to lipopolysaccharide (LPS, a mammalian B-cell mitogen used successfully with the bluegill), these results did reveal a previously unappreciated feature of fish lymphocyte responses to conconavalin A (ConA, a mammalian T-cell mitogen also used successfully with the bluegill). The temperature optimum for the pinfish mitogenic response to ConA seemingly varied depending upon the season, i.e., although responses to ConA could be obtained at lower *in vitro* temperatures during the winter months, such low-temperature *in vitro* responses were not readily apparent with fish caught during the fall or spring (3). These *in vitro* differences could result from seasonal differences in diet or reproductive state. Alternatively, they may reflect adaptation to environmental temperature. Hence the studies reported here were undertaken to

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determine the effects of *in vivo* acclimation temperature on the *in vitro* response of pinfish cells to ConA and on the physical properties of fish lymphocyte membranes using fluorescence depolarization.

Material and Methods. *Experimental animals.* The pinfish, *Lagadon rhomboides*, a euryhaline teleost and a member of the family Sparidae, was used. Animals weighing 85–200 g were caught with a hook and line from the Matanzas River and transported promptly to holding tanks at the Whitney Marine Laboratory. The holding tanks were supplied with flow-through sand-filtered fresh seawater (a complete change of water occurred at least twice daily) and were arranged into batteries of four or five tanks each. Within each battery, seawater was partially recirculated through a heat exchanger (Neslab Instruments) outfitted with either heating or cooling elements to facilitate temperature regulation. All fish were fed to satiation three times weekly with Trout Chow (Purina) and/or thawed frozen shrimp. During the first week in captivity each fish was twice exposed to formaldehyde (0.25 ml/liter for 1 hr) as a prophylactic treatment for external parasites.

Preparation of pinfish lymphocyte suspensions. Pinfish were anesthetized in a dilute solution of tricane methane–sulfonate (Crescent Research Chemicals) and exsanguinated from the caudal artery/vein. Cell suspensions from pooled spleen and anterior kidney were prepared by teasing the tissue through a 60–80 mesh wire screen. Lymphocytes were isolated from this suspension using the Böyum method (4), i.e., centrifugation at room temperature at 500*g* for 45 min over lymphoprep (Nyegaard). The interface (buffy coat) cells were judged, based upon the examination of May–Grünwald–Giemsa-stained cytofuge preparations, to contain 35–85% lymphoid cells depending on the donor fish.

In vitro mitogen stimulation of pinfish lymphocytes. The microculture system described previously by Strong *et al.* (5) was used in this study with a few modifications. The culture medium was RPMI 1640 (Grand Island Biological Co.) containing heparin and the antibiotics used previously with bluegill lymphocytes (2). This medium also contained an additional 1.76 g/liter NaCl in order to approximate the osmolarity of pinfish plasma. Culture medium was supplemented with 10% sheepshead serum. The sheepshead (*Archosargus probatocephalus*) is a relatively large member of the family Sparidae and was the only source of serum supplement found to be suitable. Suspensions of pinfish lymphocytes (5×10^5 cells/200 μl medium) were cultured in 96-well, round-bottom, plates (Linbro); ConA (lots 141 and 98, Miles-Yeda Ltd.) was added in 10 μl medium. The cultures were incubated at the desired temperatures in humidified CO_2–air incubators. The CO_2 concentration was adjusted to maintain the culture medium at pH 7.2. In the experiments reported here with cells from 17°- and 27°-acclimated fish, the cultures were incubated at 20° and 30°. These particular *in vitro* temperatures were selected because it was shown by others that fish exhibit a 3° increase in preferred water temperature (behavioral fever) when injected with pyrogens (12). It seemed reasonable, therefore, in the absence of a very large number of incubators, to monitor ConA responses 3° higher than the arbitrary acclimation temperatures. Cultures were pulsed 24 hr with tritiated thymidine (0.5 μCi/well, Schwarz/Mann) prior to harvesting. Harvesting was accomplished with a multiple automatic sample harvester (Otto Hiller Co.) using deionized water to lyse the cells and precipitate the nucleic acids onto grass-fiber disks (Reeve Angel). The disks were dried, placed into scintillation fluid, and counted for ^3H. The results from mitogen-stimulated cells are expressed as stimulation indices, i.e., *E/C*, were *E* is the mean of the counts per minute from triplicate cultures with mitogen and *C* is the mean of the counts without mitogen.

Preparation of purified plasma membranes from pinfish lymphocytes. Lymphocytes were isolated from the pooled spleens and anterior kidneys of pinfish essentially as described above. In this instance, however, all procedures were carried out using pinfish phosphate-buffered saline, pH 7.2 (PPBS). PPBS contained 10.76 g NaCl, 31 μg heparin, 9.5 ml of 0.2 *M* NaH_2PO_4, and 40.5 ml of 0.2 *M* Na_2HPO_4 per liter of double-distilled water.

Plasma membranes were purified ac-

cording to a technique described in detail by Crumpton and Snary (6). The isolated pinfish lymphocytes were washed twice in PPBS and diluted to a concentration of 1.0 to 1.5 $\times$ 10^7/ml. The cells were then passed through a hydraulic cell disrupter (Stansted Model 612) to strip the plasma membranes from the cells. Back pressure on the hydraulic pump was adjusted so that the number of viable cells, as judged by trypan blue exclusion, was reduced by about 95%. The lysed cell suspension was collected in tubes on ice and promptly centrifuged at 500*g* for 10 min to remove any large cellular debris. The supernatant was then centrifuged at 3500*g* for 15 min to remove the smaller debris. Finally, the plasma membrane fraction was pelletted by centrifugation at 30,000*g* for 40 min. The supernatant was discarded and the membrane pellet stored frozen until used.

Membrane fluidity measurements. The fluidity of the purified plasma membranes was estimated by fluorescence polarization using the fluorescence probe, 1,6-diphenyl-1,3,5 hexatriene (DPH; Aldrich) as previously described (7, 8).

Membrane pellets were resuspended in 50 m*M* Tris buffer (pH 7.5). Protein concentrations were determined by the Lowry method (9) and adjusted to 100 μg protein/ml. DPH stock solution (2 $\times$ 10^{-3} *M* in tetrahydroforan) was diluted with 50 m*M* Tris buffer (pH 7.5) to a working concentration of 2 $\times$ 10^{-6} *M* by injecting a small amount of stock solution (10 μl/10 ml Tris) into rapidly stirred buffer. Equal volumes of the DPH working solution and the membrane sample were mixed and shaken gently at 30° for 2 hr. All DPH solutions were shielded from light.

Fluorescence depolarization was measured in an SLM Polarization Fluorometer (SLM Instruments) equipped with dual photomultipliers and a water-jacketed cuvette holder. This instrument, employing the T-format design (10), allowed the simultaneous measurement of emission intensities in the vertical plane (I_V) and the horizontal plane (I_H) and automatically calculated the ratio, I_V/I_H, for each trial. The wavelength of excitation was set at 360 nm with a 2-nm bandwidth while emission was measured at wavelengths above 420 nm using a Schott cut off filter. Temperature of the fluorescent sample in the instrument was controlled by a circulating water bath (RTE-8, Neslab Instruments) and monitored by a thermocouple (YSI Telethermometer with microprobe) placed in a duplicate cuvette in the four-place cuvette holder.

Polarization values (P) and anisotropy values (r) were calculated according to

$$P = (R_V/R_H) - 1/(R_V/R_H) + 1,$$
$$r = (R_V/R_H) - 1/(R_V/R_H) + 2,$$

where R_V is the ratio, I_V/I_H, when the plane of excitation is vertical and R_H is the ratio, I_V/I_H, when the plane of excitation is horizontal. These equations take into account the grating correction factor, G, that compensates for parallel diffraction amonalies (11). Each P and r value reported represents the mean of at least 60 trials. Readings were corrected for background scatter due to intrinsic properties of the membranes themselves by blanking the fluorometer against duplicate membrane suspensions without fluorescent probe. Due to the nature of the scans in Fig. 2 these measurements were not corrected for background scatter. This caused no difficulty in interpretation since scatter in those cases where measured was found to account for no more than 3.5% of the transmitted signal. The resultant polarization values were therefore only slightly lower than those obtained when readings were corrected for background scatter.

Results. *Effect of acclimation temperature of pinfish on in vitro lymphocyte responses.* The preliminary observations (3) that the optimum temperature for *in vitro* responsiveness of pinfish lymphocytes decreased during the winter prompted the hypothesis that fish acclimated to different environmental temperatures may yield lymphocytes that have different *in vitro* temperature optima for ConA responsiveness. This hypothesis was tested in fish acclimated to either 17° or 27° for at least 3 weeks prior to use. Lymphocyte suspensions from the anterior kidneys and spleens of individual fish were cultured *in vitro* at 20° and 30° with varying concentrations of ConA. The results from two different experiments wherein pairs of fish (one 27° and

one 17° acclimated) are compared are depicted in Fig. 1. It can be seen that at an *in vitro* temperature of 30° the ConA responses of cells from 17° and 27° acclimated fish were substantial. However, at an *in vitro* temperature of 20°, two major differences were seen. First, lymphocytes from 17° acclimated fish had much higher stimulation indices after 5–7 days in culture with ConA than did cells from 27° acclimated fish. Second, the ConA dose responses of the two fish were quite different. These results, also seen with two other pairs of acclimated fish, support the contention that the *in vivo* acclimation temperature of a fish can influence the response of its lymphocytes in culture.

Membrane fluidity of pinfish lymphocytes. Since mitogen stimulation of lymphocytes is a membrane-mediated event, the effects of temperature on lymphocyte membranes were investigated. Purified lymphocyte plasma membranes were prepared from groups of 12 fish acclimated to either 27° or 17° for at least 2 weeks and from a group of 12 fish held in ambient seawater (19–20°). The fluidities of these three membrane preparations were estimated by the fluorescence polarization. The results presented in Table I show that lymphocyte membranes from 27° acclimated fish were more viscous (have higher P and r values) than lymphocytes from 17° acclimated fish when both were measured at the same *in vitro* temperature. However, the fluidity of 27° acclimated fish, measured at 27°, was nearly identical to the fluidity of 17° acclimated fish measured at 17°. Thus it appears that homeoviscous adaptation (13) to environmental temperature occurs in the membranes of fish immune cells.

Complete temperature profiles of the fluorescence polarization of the three membrane samples are shown in Fig. 2. The fluidities of all three samples were measured over a 10–37° temperature range and plotted against the inverse of the absolute temperature. As expected, membranes from the warmest animals had the greatest viscosity at all temperatures tested. As the membranes were cooled *in vitro* the viscosity of all three samples increased. Interest-

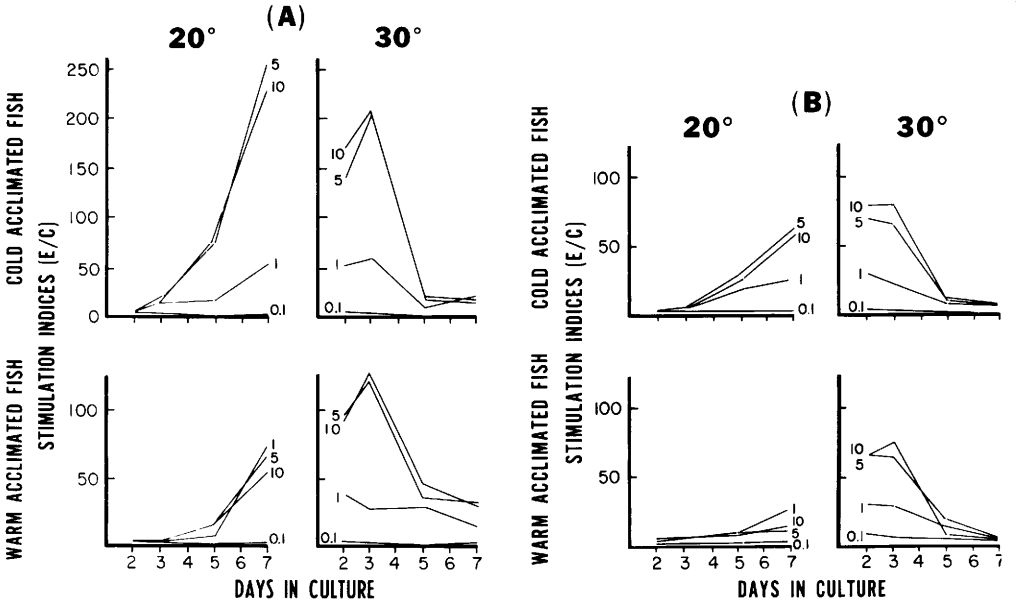

FIG. 1. Effects of *in vivo* acclimation temperature on *in vitro* responses of pinfish lymphocytes to ConA. The cold fish were acclimated to 17° for 2 weeks prior to sacrifice. The warm fish were acclimated to 27° for the same period of time. The numbers refer to the doses of ConA per culture (μg/well). A and B represent experiments with two different pairs of fish performed at different times.

TABLE I. POLARIZATION OF DPH INCORPORATED INTO LYMPHOCYTE PLASMA MEMBRANES OF PINFISH ACCLIMATED TO DIFFERENT ENVIRONMENTAL TEMPERATURES

Acclimation temperature of pinfish[a] (°)	Polarization (*P*)		Anisotropy (*r*)	
	At 17°	At 27°	At 17°	At 27°
17	0.352[b]	0.311	0.266	0.232
Ambient 19–20[c]	0.366	0.323	0.279	0.242
27	0.387	0.355	0.296	0.268

[a] Pinfish were acclimated to the indicated temperatures for at least 2 weeks prior to sacrifice. Lymphocyte plasma membranes were isolated from the pooled anterior kidney and spleen lymphocytes of about 12 fish at each acclimation temperature.

[b] *P* and *r* values are given as the mean of at least 60 trials. The maximum standard deviation for any of these data was ±0.0008.

[c] These fish were not acclimated in the laboratory. Ambient seawater temperature at the time of sacrifice was 19–20°.

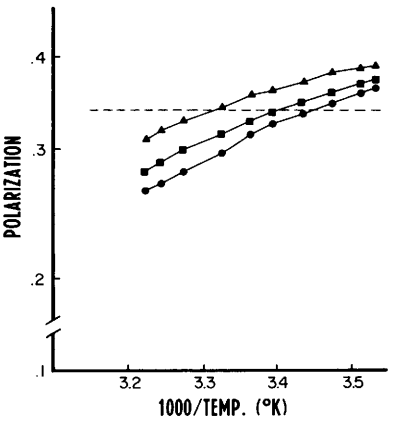

FIG. 2. The polarization of DPH incorporated into lymphocyte plasma membrane preparations of pinfish acclimated to different environmental temperatures. Membranes were isolated from fish acclimated to 27° (▲) and 17° (●). Membranes from fish held in ambient seawater (19–20°) were also tested (■).

ingly, the points on each curve corresponding to the acclimation temperature of the donor fish form a straight, horizontal line. It would seem, then, that fish are able to control the viscosity of their lymphocyte membranes at different environmental temperatures by changing the physical properties of the membranes. Thus, the influences of temperature and membrane structure interact so that a remarkably constant fluidity is maintained.

The effect of in vivo temperature downshift on membrane fluidity. The apparent homeoviscous adaptation of lymphocyte membranes, coupled with the temperature dependencies of mitogen-induced lymphocyte proliferation, prompted the theory that homeoviscous adaptation must be achieved by the immune cells before normal lymphocyte function can occur. Thus, it becomes important to establish the time course for this process. Toward this end, 28 pinfish were acclimated to a seawater temperature of 26° for 3 weeks. On Day 0, the fish were abruptly transferred to 17° seawater. Just prior to this transfer and periodically thereafter (as indicated in Fig. 3) 4 fish were sacrificed. Lymphocyte plasma membranes were purified from the pooled anterior kidneys and spleens of each group of 4 fish as described above. The fluidities of these membranes preparations were estimated by the fluorescence polarization of DPH and are presented as polarization (*P*) values. The results show that the

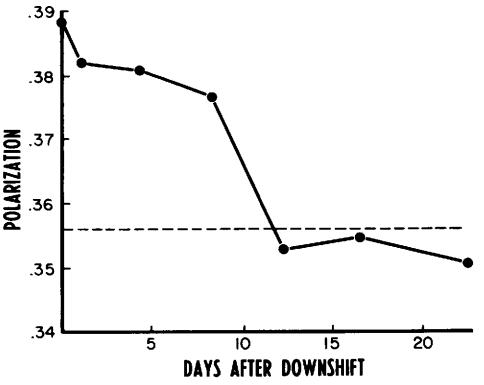

FIG. 3. Effect of a rapid temperature downshift on pinfish lymphocyte plasma membrane fluidity. The dashed line represents fluidity measurements of the membranes of fish acclimated to 26° (measured at the acclimation temperature) just prior to downshifting to 17°. The solid line corresponds to fluidity measurements on membrane preparations made from the downshifted fish at various times after the downshift (all measured at 17°).

lymphocyte membrane fluidity of the downshifted fish (measured at 17°) began to approach the fluidity established for the lymphocytes of 26° acclimated fish (measured at the acclimation temperature) as early as 1 day after the downshift. However, these values did not achieve parity until Day 12. Subsequent fluidity measurements were not appreciably different from the Day 12 values.

Discussion. The studies reported here were undertaken to gain an understanding of the mechanisms operative in the different effects of changing environmental temperature on the immune responses of ectothermic animals. These studies were based, in part, on previous observations linking temperature adaptation in ectotherms to changes in fatty acid metabolism and the subsequent incorporation of fatty acids into biological membranes. Both eucaryotes and procaryotes (14) incorporate increasing proportions of saturated and long-chain fatty acids into the membrane phospholipids as the growth temperature is increased. In bacteria this compositional variation resulted in membranes of identical viscosities at the growth temperature of the cells and has been termed homeoviscous adaptation (13).

In fish, similar studies on the membranes of gill mitochondria (15) and brain tissue (16, 17) in several species have demonstrated a general trend toward reduced fatty acid saturation at low environmental temperature. It has been suggested that this represents an adaptation toward the maintenance of an optimal membrane fluidity. For brain synaptosomal membranes, it has been concluded that this homeoviscous compensation is an important component of temperature adaptation, although only a partial temperature compensation is achieved (16, 18).

The present study shows that the temperature to which a pinfish is acclimated *in vivo* affects the response of its lymphocytes to mitogenic stimulation at a given *in vitro* temperature (Fig. 1). It is also clear that acclimation temperature influences the physical properties (i.e., fluidity) of the lymphocyte plasma membranes of the fish (Fig. 2). Since the activation of lymphocytes appears to be initiated by the binding of a ligand to the plasma membrane (19), it is likely that the effects of acclimation temperature on mitogenic stimulation of lymphocytes is due, at least in part, to its influences on the lymphocyte plasma membranes.

This conclusion with fish lymphocytes is consistent with studies in mammalian systems. The studies of Maccecchini and Burger (20) using murine lymphocytes showed that the optimum temperature for mitogen stimulation by ConA was dependent on the degree of saturation of the fatty acids incorporated into the membrane phospholipids. As the fatty acid saturation of the membranes was decreased, albeit artificially, the temperature for the optimum proliferative response to ConA was also decreased. It was concluded from preliminary fluidity studies on these altered membranes that the fluidity of the membrane lipid phase plays a role in the process of lymphocyte stimulation by lectins. Similarly there is a growing body of literature indicating that the thermodynamic state of the membrane (reviewed (21)) plays an important role in a number of other physiological functions and properties associated with membranes, such as membrane bound enzyme kinetics (22, 23), transport of low-molecular-weight substances (21) and even lymphocyte activation (24). Thus it seems reasonable to conclude that the maintenance of an optimal membrane fluidity, as achieved by homeoviscous adaptation, is important, if not crucial, to normal lymphocyte function in ectotherms, such as fish.

Future studies employing separated lymphocyte subpopulations, known to exist in fish (2, 25), are required to definitively assign the role(s) of homeoviscous adaptation on the various parameters of the immune response in fish. It would appear, however, that a model system wherein the immune response can be manipulated by a process as nonintrusive, and as seemingly reversible, as changing *in vivo* (and perhaps *in vitro*) temperatures has great potential as a vehicle for studying basic cellular interactions in an immune response. For example, the importance of time of acclimation is seemingly not seriously considered in many

experiments involving low-temperature immunosuppression in fish; the potential interrelationships between putative carrier-specific helper and suppressor cells in fish (1) need to be approached in this respect.

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