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DIFFERENTIAL TEMPERATURE ACCLIMATIZATION RESPONSES IN THE MEMBRANE PHOSPHOLIPIDS OF *POSTHODIPLOSTOMUM MINIMUM* AND ITS SECOND INTERMEDIATE HOST, *LEPOMIS MACROCHIRUS*

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ABSTRACT: The effects of temperature change on phospholipid content in metacercariae of *Posthodiplostomum minimum* and their second intermediate hosts, *Lepomis macrochirus*, were examined to gauge similarities in the homeoviscous adaptation of host and parasite membranes to environmental thermal change. Heart, liver, and muscle tissues from individual *L. macrochirus* responded to environmental temperature declines with a decrease in the ratio of phosphatidylethanolamine (PE) to phosphatidylcholine (PC). Increases in membrane PE concentration increase membrane fluidity, maintaining fish membrane function as environmental temperature declines. However, the metacercariae of *P. minimum* exhibit changes in cholesterol levels, total lipid levels, and lipid composition (PE/PC) that contrast the normal changes for homeoviscous membrane adaptation exhibited by their fish intermediate hosts. The parasites seem to rely on their hosts for homeoviscous adaptation within normal developmental temperature ranges, pooling both cholesterol and PE as energetic stores for development and ontological transitions signaled by elevated temperatures.

Posthodiplostomum minimum is among the most widely reported larval digenean parasitizing freshwater fishes. Adult worms occur in birds, snails are used as first intermediate hosts, and a variety of freshwater fishes serve as second intermediate hosts. In the bluegill sunfish, *Lepomis macrochirus*, metacercariae of *P. minimum* are found in the supporting tissues and mesenteries around the heart, liver, air bladder, and other viscera. The ready availability of *L. macrochirus* and the ubiquity of *P. minimum* combine with high prevalence and intensity levels to produce an excellent model system for cellular studies of host–parasite biology, particularly lipid biochemistry. The study presented here examines the effect of temperature change on phospholipid and cholesterol content in both metacercariae of *P. minimum* and the heart, liver, and muscle tissue of their second intermediate host, *L. macrochirus*, to gauge similarities in the physiological membrane acclimatization of host and parasite membranes in response to temperature change.

The structure and function of a biological membrane are influenced both by temperature and lipid composition. Membrane lipids exist in a liquid crystalline bilayer state, maintaining the balance between membrane fluidity and rigidity. In vitro, membrane phase structure varies with temperature, shifting from a gel phase at low temperatures to a more fluid liquid crystalline bilayer phase at higher physiological temperatures, and finally an inverted lipid structure called the hexagonal (H_{II}) phase (March, 1990; Tenchov, 1991; Seddon et al., 1997). However, membrane structure in vivo is mediated by lipid composition, specifically, the relative proportions of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (often expressed as a PE/PC ratio). The PE/PC ratio determines bilayer phase structure, and, therefore, virtually all functional properties of the membrane.

PC is cylindrical and favors the formation of bilayer-shaped gel phases, but PE is conical, favoring the formation of H_{II} phases (Cullis et al., 1980; Marsh, 1990) (Fig. 1). Both structural phases readily form with a well defined transition temperature in in vitro aqueous systems by using pure phospholipids (Silvius, 1982). Living membranes are mixed systems that use predominantly PC and PE, mediating their relative proportions to alter membrane structure and function. (For the sake of clarity, ''phospholipid'' is used here to mean structures with a phosphate linkage [Fig. 1] whereas ''lipid'' includes both phospholipids and cholesterols.) A membrane's PE/PC is directly correlated to membrane structure and thus regulates membrane function. As the PE/PC ratio increases, the H_{II} phase begins to develop in select regions of the bilayer called ''rafts'' (Simons and van Meer, 1988; Cullis, 1980; van Meer, 2000; Rajendran and Simons, 2005) (Fig. 2). These rafts are associated with membrane fusion events such as exocytosis (Paavo and Kinnunen, 1992) and endocytosis (Parton and Richards, 2003) as well as a general increase in the fluidity within a membrane (Shukla et al., 1980; Hayakawa et al., 1998).

For poikilothermic animals, shifts in PE/PC can be used to shift membrane structure, maintaining membrane fluidity and function in the face of a variable environment. Such organismal mediation of lipid composition in response to cold acclimation is called homeoviscous adaptation. Most studies of homeoviscous adaptation use fish models, and increases in PE/PC in response to declining temperature have been demonstrated for a variety of agnathan, elasmobranch, and various teleost fishes (Hazel and Williams, 1990; Hazel, 1995; Glemet and Ballantyne, 1996; Hazel et al., 1998; Brooks et al., 2002). Although Barrett et al. (1970) and Fried and Shapiro (1979) reported phospholipid compositions for *Echinostoma trivolvis* and Yusufi et al. (1976) reported phospholipid compositions for *Echinostoma malayanum*, no previous study has examined the physiological ability of or necessity for parasites to modify lipid content for homeoviscous adaptation.

MATERIALS AND METHODS

Sample collection and lipid extraction

Nine adult *L. macrochirus* were collected by hook and line from Verdon Lake, Richardson County, Nebraska. Lake temperature ranged from 8 to 14 C. Fish were transported in aerated lake water, randomly assigned to treatment groups of 3 individuals each, and housed by treatment group in separate containers. Water temperature in each container was slowly equilibrated to 4, 14, or 21 C over a 24-hr period by using large ambient temperature and ice-chilled water baths.

After 24 hr, fish were killed and samples of liver (0.61–0.84 g wet weight), heart (0.09–0.14 g wet weight), and muscle tissue (0.43–0.58 g wet weight) were collected from each individual, transferred to Locke's solution held at the appropriate treatment temperature, and ho-

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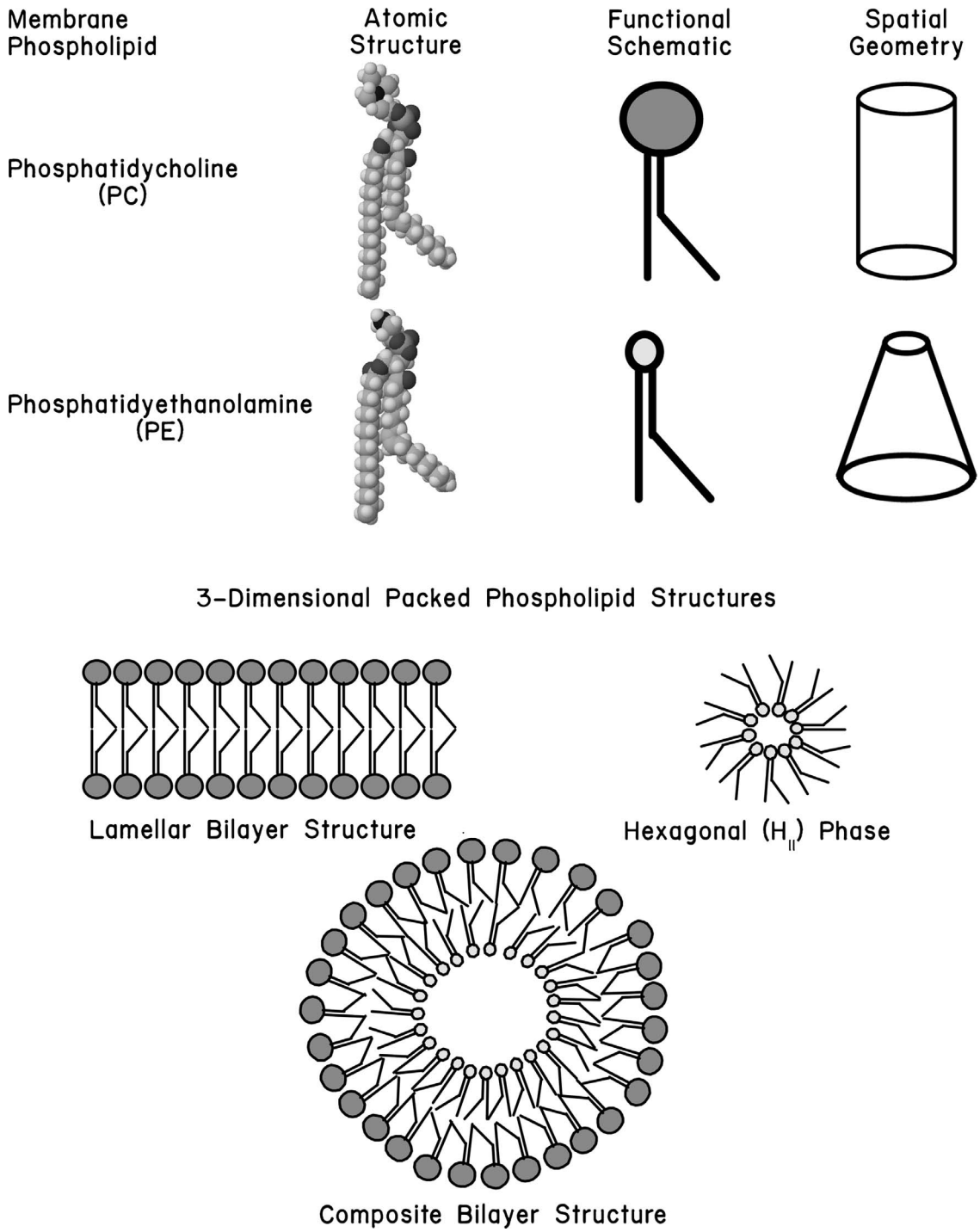

FIGURE 1. Atomic structure, functional schematic, spatial geometry of 2 membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (top), and their resulting 3-dimensional packed structural arrangements. Both PC and PE are made up of head groups (PC head group, PO_4-$CH_2CH_2N(CH)_4^+$; PE head group, PO_4-$CH_2CH_2NH_4^+$) attached to the hydrophobic tail through a phosphate (PO_4) bond. Methyl groups present in PC significantly increase the size of the head group, and, therefore, the 3-dimensional packing order of the molecule: spatially, PC is cylindrical, but PE resembles a truncated cone. In the absence of PE, PC forms the classical lamellar bilayer structure(center, left), but as membrane lipid composition approaches a 3:1 ratio of PC to PE, differences in head group spacing produce a composite bilayer structure with a PC-rich outer sheaf and a PE-rich inner sheaf (bottom). When excess PE is present, it is removed from the membrane through formation of independent H_{II} phase structures (center, right).

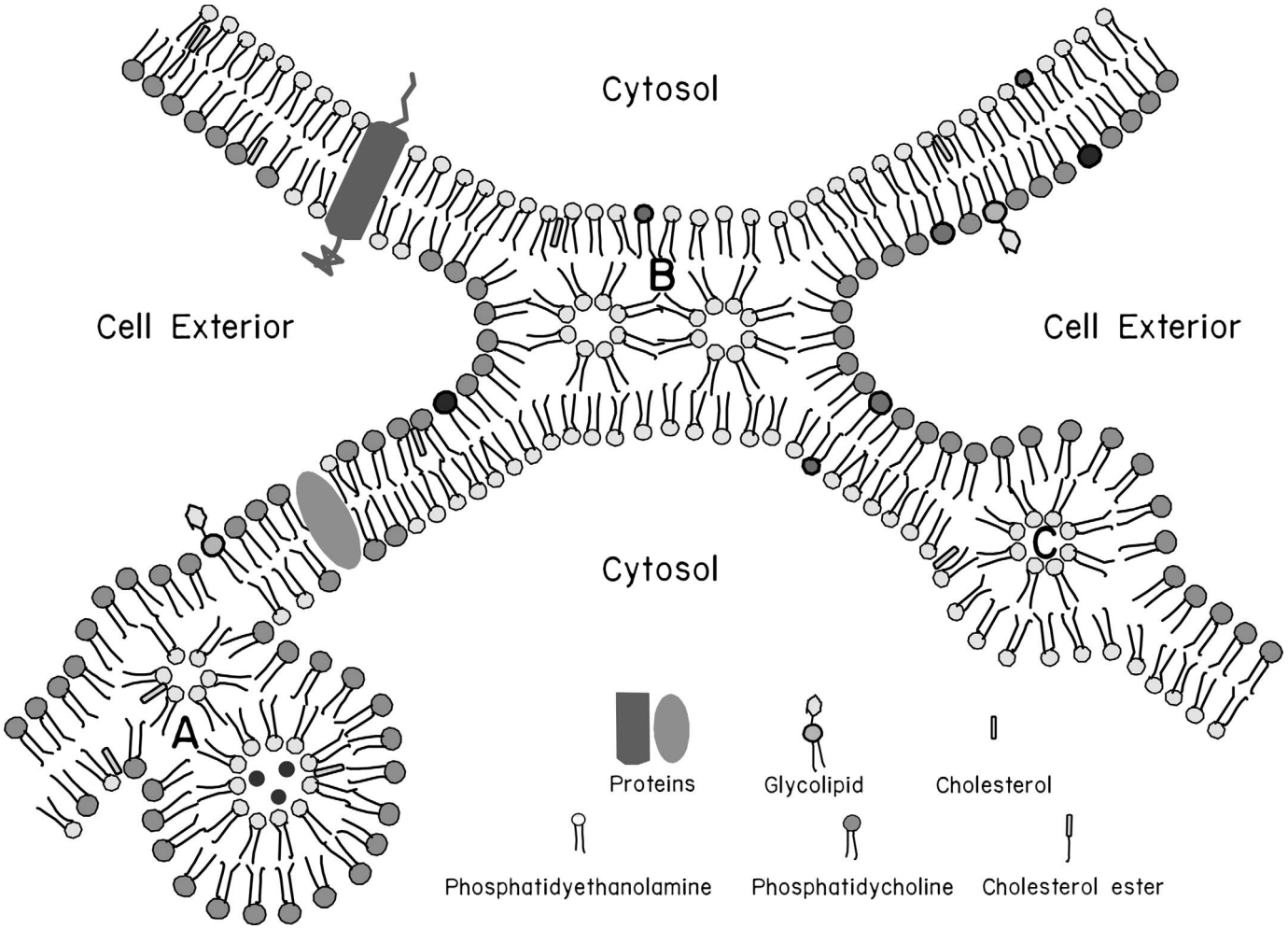

FIGURE 2. Metamorphic mosaic model of biological membranes. Local differences in PE/PE composition produce membrane structure facilitating a variety of membrane functions, including exocytosis fusion (**A**), a hexagonal phase structure permitting the semifusion of 2 bilayers (**B**), and a H_{II} phase ''raft'' allowing polar molecules to access the membrane (**C**).

mogenized in a 10-ml Broeck tissue grinder. Lipids were extracted by the procedure of Bligh and Dyer (1959). Methanol and chloroform were added to each homogenate stepwise to produce a final ratio of 3:2:1 (typically 6 ml of chloroform and 4 ml of methanol to 2 ml of homogenate). The organic lipid layer was removed; 6 ml of chloroform was added stepwise and extracted similarly 2 more times before pooling all organic extractions from a single sample. A fourth chloroform extraction conducted on a liver sample resulted in only an additional 0.5% lipid. Each pooled organic extraction was back-extracted twice with 5 ml of distilled water, dried by rotoevaporation, and resuspended in 500 μl of chloroform.

Three hundred metacercariae (0.146–0.172 g wet weight) were removed from the air sac of each fish, pooled, and rinsed in Locke's solution held at the appropriate treatment temperature. Each pooled sample of metacercariae was homogenized, and their lipids were extracted using the methods described above.

Lipid separation, identification, and quantification

Aliquots from sample extractions were analyzed for total phospholipids content by using the ammonium ferrothiocyanate method of Stewart (1980). Phospholipids and cholesterol were separated by thin layer chromatography on plastic sheet silica gel 60 plates that had been activated for 40 min at 110 C as follows: 400 nmol of total phospholipids was placed on a plate and separated with a mobile phase containing 65 parts chloroform, 25 parts methanol, and 10 parts acetic acid (Barry Ganong, pers. comm.). After separation, silica plates were dried and exposed to iodine vapors to visualize lipid groups. The following R_f values were measured for a mixture of lipid standards: PC = 0.128, phosphatidylserine (PS) = 0.288, PE = 0.478, phosphatidylglycerol = 0.584, and cholesterol = 0.913. Phospholipids were mutually exclusive with the smallest degree of separation, which was between PC and PS, being 0.55 cm. Cholesterol and cholesterol-palmitate were not separated. Silica scrapings containing isolated lipids were removed and extracted 3 times with 2 ml of 4 parts chloroform: 1 part methanol. Samples were dried by rotoevaporation and analyzed for phospholipids content as described previously. Cholesterol content was determined using a commercially available cholesterol reagent kit (Point Scientific Inc., Lincoln Park, Michigan).

RESULTS

Total lipid content of *P. minimum* and *L. macrochirus* tissue samples are presented in Figure 3. The variation of total lipid content for all *L. macrochirus* tissues tested were similar showing a significant decrease in content between 14 and 21 C. *P. minimum* demonstrated a rapid increase in lipid content between 4 and 14 C followed by a dramatic decrease between 14 and 21 C.

Host and parasite display distinctly different trends in lipid composition over temperature (Fig. 4). The PE/PC ratios of all

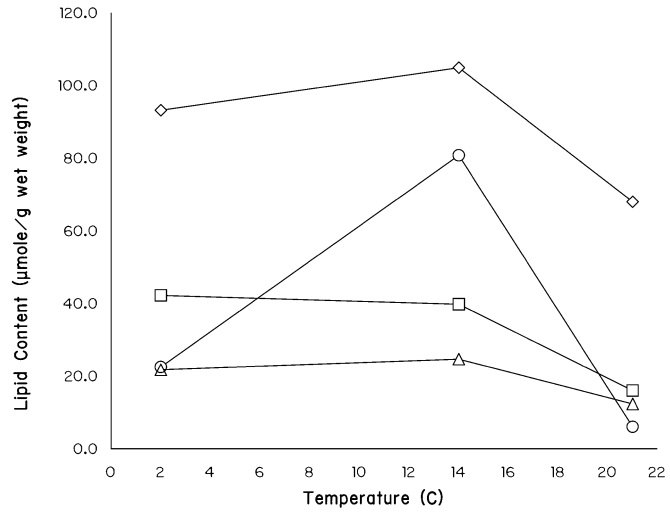

FIGURE 3. Trends in phospholipid (noncholesterol) content of host heart (□), muscle (△), liver (◇), and metacercariae (○) tissue between 2 and 21 C.

host tissue types decreased with increasing environmental temperature, structurally reducing membrane fluidity and counteracting the expected increases in membrane fluidity at higher physiological temperatures. In contrast, the PE/PC ratio of parasite tissue shows a remarkable increase with increasing environmental temperature.

A more complete evaluation of the effect of a PE/PC composition shift in a biological membrane depends upon the relationship of PE and PC content in relationship to total phospholipid content (PE, PC, PS, and phosphatidylgycerol). The distribution of phospholipids in biological membranes is not symmetrical (Devaux and Zachowski, 1994), and transverse diffusion between outer and inner leaflets is a very slow process (Kornberg and McConnell, 1971). Phosphatidylcholine resides almost exclusively in the outer leaflet, whereas PE resides in the inner leaflet of a membrane, producing a rigid, less interactive exterior leaflet and a more fluid, interactive leaflet bounding the cytoplasm. Bluegill sunfish tissues increase overall PC levels and decrease overall PE levels in response to increased temperatures (Table I). The conversion of total lipids to PC is sufficient at 14 C to force the insertion of PC into the inner leaflet, producing a more rigid membrane and slowing cellular metabolism. In contrast, the parasite seems to be converting PE to PC or preferentially sequestering PE rather than PC from host tissues.

TABLE I. Percentage of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) making up total phospholipid content for host heart, muscle, heart, and metacercarial tissues acclimated at 4, 14, and 21 C.

	PE/total phospholipid*			PC/total phospholipid		
Tissue	4 C	14 C	21 C	4 C	14 C	21 C
Host heart	42.7	28.3	18.5	42.3	59.1	71.9
Host muscle	36.6	31.0	11.1	42.7	65.8	38.8†
Host liver	30.1	22.0	20.8	51.2	59.5	70.8
Metacercaria	16.9	16.2	36.6	61.7	61.8	43.3

* Total phospholipid content includes PC, PE, phosphatidylserine, and phosphatidylglycerol.
† Muscle tissue at 21 C contains 43.6% phosphatidylserine.

Again, there are differences in host tissue and parasite response as revealed by cholesterol analysis. Host tissues increase in cholesterol content as the temperature both increases and decreases (Fig. 5), but the parasite displays a direct relationship between cholesterol content and increasing environmental temperature.

DISCUSSION

Bluegill sunfish undergo homeoviscous adaptation in a manner similar to that reported for other lake fish species. Colder temperatures facilitate an increase of PE with a corresponding loss of PC (Fig. 4; Table I). These head group changes have been reported to occur within hours of the onset of thermal changes (Hazel and Landrey, 1988a, 1988b; Carey and Hazel, 1989). In contrast, a polyunsaturation shift of the fatty acid tails

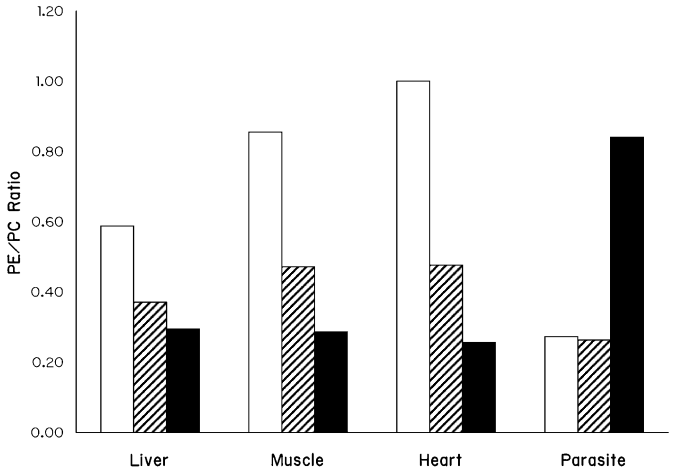

FIGURE 4. Ratio of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in host liver, muscle, heart, and metacercariae tissues maintained at 2 C (open bars), 14 C (shaded bars), and 21 C (solid bars).

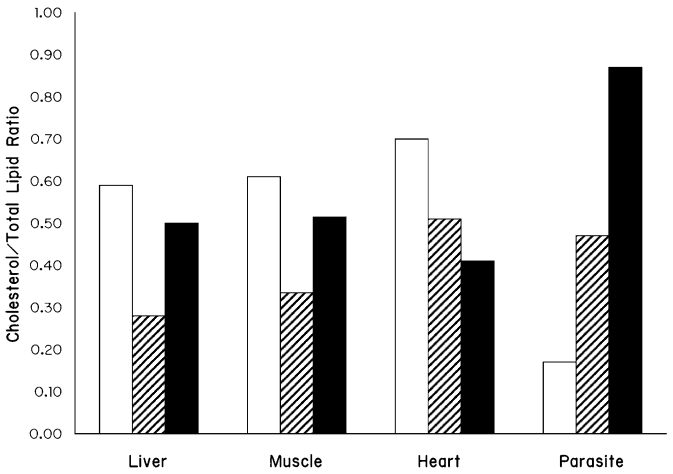

FIGURE 5. Ratio of cholesterol to total lipid content in host liver, muscle, heart, and metacercariae tissues maintained at 2 C (open bars), 14 C (shaded bars), and 21 C (solid bars).

requires weeks (Sinensky, 1974; Guderley and St-Pierre, 2002). The ability of PE to destabilize a membrane and increase its fluidity accommodates membrane function and thus cellular function at low temperature. Increases in cholesterol levels at high and low temperatures also are consistent with homeoviscous adaptations to maintain homeostatic membrane function. Cholesterol extends the window for membrane phase transition to a wider range of temperature by stiffening them at warmer temperatures but loosening them at lower temperatures (Yeagle et al., 1990). Therefore, cholesterol allows membrane fluidity to be less sensitive to sudden environmental temperature change, in effect stabilizing membrane fluidity by mediating sharp phase transitions. Given these membrane stabilizing mechanisms, decreases in phospholipid content with increasing temperature are not surprising. The 10-fold elevation in of phospholipid content in the liver relative to heart and muscle tissues simply reflects the liver's function as the primary site of lipid production.

In contrast to their hosts, it seems that metacercariae of *P. minimum* are incapable of homeoviscous membrane adaptation. They do not facilitate the appropriate phospholipid head group changes, and within normal developmental temperature ranges they may depend upon the host to mediate temperature changes. The dramatic change in metacercaria lipid content in response to temperature does not provide membrane homeostasis. These changes may be related to ontogeny rather than membrane structure and function. Most metacercaria lipids are localized in the fluid separating the metacercaria from the enclosing cyst wall. There is evidence that metacercariae can catabolize their lipid stores (Barrett, 1977), suggesting that metacercaria lipid pooling between 2 and 14 C may reflect the creation of an energy stockpile for development and ontological transition. Alternatively, lipid content is also correlated to the onset of host immune response (Barrett, 2000) and lipid pooling may be related to the retention of immunogenic compounds. These strategies are consistent with the continued growth and maintenance of the metacercaria in the fish intermediate host. Elevated changes in temperature (the shift from 14 to 21 C) may signal a seasonal change or transmission event that elicits a preparatory physiological response from metacercariae in which they begin to pool PE and cholesterol in preparation for excystation and ontological transition. In contrast to their hosts, metacercariae of *P. minimum* increase or pool their cholesterol content as temperature increases to 21 C. Metacercaria lipid pooling at higher temperatures is not a reflection of increased lipid availability in the host. During this temperature transition, host tissues are actively metabolizing cholesterol for homeoviscous membrane adaptation; thus, if anything, available host lipids are more scarce at increasing temperatures.

In response to environmental temperature change, the metacercariae of *P. minimum* exhibit changes in cholesterol levels, total lipid levels, and lipid composition (PE/PC) that contrast the normal changes for homeoviscous membrane adaptation exhibited by their fish intermediate hosts. The parasites seem to rely on their hosts for homeoviscous adaptation within normal developmental temperature ranges, pooling both cholesterol and PE as energetic stores for the development and ontological transitions signaled by elevated temperatures.

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