Journal of Aquatic Animal Health 21:262–271, 2009

DOI: 10.1577/H07-056.1

[Article]

Linking Egg Thiamine and Fatty Acid Concentrations of Lake Michigan Lake Trout with Early Life Stage Mortality

SERGIUSZ CZESNY* AND JOHN M. DETTMERS[1]

University of Illinois, Illinois Natural History Survey, Lake Michigan Biological Station, 400 17th Street, Zion, Illinois 60099, USA

JACQUES RINCHARD[2] AND KONRAD DABROWSKI

School of Environment and Natural Resources, The Ohio State University, Columbus, Ohio 43210, USA

Abstract.—The natural reproduction of lake trout *Salvelinus namaycush* in Lake Michigan is thought to be compromised by nutritional deficiency associated with inadequate levels of thiamine (vitamin B_1) in their eggs. However, mortality driven by thiamine deficiency (commonly referred to as early mortality syndrome [EMS]) is not the only significant cause of low lake trout survival at early life stages. In this study, we sought to better understand the combined effects of variable levels of thiamine and fatty acids in lake trout eggs on prehatch, posthatch, and swim-up-stage mortality. We sampled the eggs of 29 lake trout females from southwestern Lake Michigan. The concentrations of free thiamine and its vitamers (e.g., thiamine monophosphate [TMP] and thiamine pyrophosphate [TPP]) as well as fatty acid profiles were determined in sampled eggs. Fertilized eggs and embryos were monitored through the advanced swim-up stage (1,000 degree-days). Three distinct periods of mortality were identified: prehatch (0–400 degree-days), immediately posthatch (401–600 degree-days), and swim-up (601–1,000 degree-days). Stepwise multiple regression analysis revealed (1) that *cis*-7-hexadecenoic acid in both neutral lipids (NL) and phospholipids (PL) correlated with prehatch mortality, (2) that docosapentaenoic acid in PL and docosahexaenoic acid in NL correlated with posthatch mortality, and (3) that total lipids, TPP, and palmitoleic acid in NL, linoleic acid, and palmitic acid in PL correlated with the frequency of EMS. These results indicate the complexity of early life stage mortality in lake trout and suggest that inadequate levels of key fatty acids in eggs, along with variable thiamine content, contribute to the low survival of lake trout progeny in Lake Michigan.

A self-sustaining status for the Lake Michigan populations of lake trout *Salvelinus namaycush* is a primary but unmet goal of fisheries managers. Historically, the lake trout thrived in its native habitats of Lake Michigan, constituting one of the largest fisheries for this species in the world. By the early 1950s, however, a combination of overfishing, predation by sea lampreys *Petromyzon marinus*, the invasion of alewives *Alosa pseudoharengus*, and habitat degradation caused this native predator to become nearly extinct (Hile et al. 1951; Eschmeyer 1957). Since then, large numbers of hatchery-origin lake trout have been stocked every year in an effort to reestablish natural recruitment in Lake Michigan. Although these fish generally survive to adulthood and some are capable of producing viable eggs, no significant natural recruitment has been recorded. Bronte et al. (2008) provides an extensive summary of rehabilitation efforts while highlighting impediments to the restoration of lake trout in Lake Michigan. It appears that early mortality syndrome (EMS) and predation on eggs and alevins, along with lakewide population levels that are too low and improper stocking practices are contributing to the lack of natural recruitment of lake trout. Thus far, no single factor has been identified as the prime hindrance to natural recruitment.

Nutritional deficiencies associated with inadequate levels of thiamine (vitamin B_1) in the eggs have resulted in high mortalities at the swim-up stage of several salmonid species in the Great Lakes (Marcquenski and Brown 1997; Brown et al. 1998a), New York's Finger Lakes (Fisher et al. 1995), and the Baltic Sea (Amcoff et al. 1998). Mortality caused by thiamine deficiency, commonly referred to as EMS, is a likely effect of the maternal diet's containing a large proportion of prey fish with high levels of thiaminase, an enzyme that degrades thiamine. High levels of thiaminase are found in forage species such as alewives and rainbow smelt *Osmerus mordax* (Tillitt et al. 2005). Thus, EMS may be a significant bottleneck to

* Corresponding author: czesny@illinois.edu

[1] Present address: Great Lakes Fishery Commission, 2100 Commonwealth Boulevard, Suite 100, Ann Arbor, Michigan 48105, USA.

[2] Present address: Department of Environmental Science and Biology, State University of New York, the College at Brockport, 350 New Campus Drive, Brockport, New York 14420, USA.

Received December 26, 2007; accepted January 5, 2009
Published online December 14, 2009

lake trout recruitment (Brown et al. 2005b) because alewives are a major component of their diet in Lake Michigan (Madenjian et al. 1998).

Lake trout offspring affected by EMS die at the swim-up stage (anywhere between 600 and 1,000 cumulative degree-days after fertilization). However, significant mortality at earlier life stages (embryonic and immediately after hatch) has also been observed. Blue sac disease, which induces mortality after hatch but before the onset of EMS, has been linked to dioxin exposure. Exposure to dioxins results in oxidative stress associated with the peroxidation of lipid membranes and leads to edema, hemorrhaging, and ultimately mortality (Cantrell et al. 1998). Of all lipids, polyunsaturated fatty acids, which are major components of all biomembranes, are especially susceptible to the oxidative action of free radicals and thus a leading factor in the deterioration of membrane functions.

Lipids, particularly polyunsaturated fatty acids (PUFAs), have been recognized to play important roles in regulating and maintaining the overall fitness of fish and population stability (Adams 1998). Fish eggs contain high levels of PUFAs that later, during embryonic development, provide adequate biomembrane fluidity (Hazel 1989). A high level of PUFAs in phospholipids is a critical adaptation in ectotherms because low temperatures could hinder membrane function and cellular metabolism. Several studies have documented that the successful reproductive performance of a fish population is strongly linked to the availability of lipid reserves for gonad development. Lipid energy stored in the livers of mature female Atlantic cod *Gadus morhua* in the Barents Sea was used as a predictor of their reproductive potential and recruitment (Marshall et al. 1999). It is well established in teleost fish that PUFAs of both the omega-6 (n-6) and omega-3 (n-3) families, that is, arachidonic acid (AA; 20:4[n-6]),[3] eicosapentaenoic acid (EPA; 20:5[n-3]), and docosahexaenoic acid (DHA; 22:6[n-3]) are required to ensure optimal survival, growth, and development (Watanabe et al. 1982; Sargent et al. 1989; Sargent 1995).

Newly hatched fish are especially vulnerable to deficiencies associated with inadequate amounts of PUFAs in their yolk reserves because their rapidly developing tissues, especially the nervous system (including the eyes), requires large quantities of these fatty acids for proper functioning. Thus, any deficiencies, imbalance, or disturbance in the synthesis of fatty acids in the yolk and in the developing embryo may contribute to increased mortality before or shortly after hatch. In rainbow trout *Oncorhynchus mykiss* broodstock, a diet deficient in n-3 fatty acids decreased the number and size of eggs (Watanabe et al. 1984), increased the incidence of early embryonic mortality, and caused physiological dysfunction of the developing fish (Watanabe et al. 1982). On the contrary, high levels of dietary n-3 PUFAs were also associated with reduced egg production and caused yolk sac hypertrophy and decreased survival in gilthead bream *Sparus auratus* larvae (Fernandez-Palacios et al. 1995). The negative effects of both high and low levels of n-3 PUFAs indicate that an intermediate level is required to ensure the best reproductive responses. In earlier work, we have demonstrated that proper DHA:EPA and n-3:n-6 PUFA ratios are important factors in the survival of larval walleyes *Sander vitreus* (Czesny et al. 1999).

Adequate levels of fatty acids in the cellular membranes of embryos and early life stages of developing fish depend on yolk reserves and the rate of fatty acid synthesis. As a coenzyme in energy metabolism, thiamine regulates the rate of synthesis of acetyl-CoA carboxylase and fatty acid synthetase, the two enzymes directly involved in fatty acid synthesis. Therefore, it is important to investigate the variability of thiamine and fatty acids together in the context of the early survival of lake trout. A correlation between PUFAs and M74 syndrome (equivalent to EMS in the Baltic Sea) was identified in Atlantic salmon, with marked differences in the fatty acid content of healthy and M74-affected eggs (Pickova et al. 1998). Therefore, the aim of this study was to investigate the individual variation in the fatty acid and thiamine contents of lake trout eggs and to correlate that with the mortality of offspring at three distinct developmental stages. This will lead to better understanding of the effects of the above-mentioned nutrients on the frequency of mortality in lake trout offspring from fertilization to advanced swim-up.

Methods

Lake trout gamete collection, fertilization, and incubation.—Lake trout were captured with gill nets set by the Illinois Department of Natural Resources (IDNR) in the southwestern part of Lake Michigan during November 2003. Fish were caught on the Waukegan Reef (6 mi east of Waukegan Harbor [1 mi = 1.61 km]), Julian's Reef (9 mi southeast of Waukegan Harbor), and "Wire Mill" (1 mi southeast of Waukegan Harbor). All these sites are located within a 15-mi radius and were not considered separately in this study. Sperm was obtained from several males and

[3] In the terms such as 20:4(n-6), the number to the left of the colon is the number of carbon atoms in the compound, the number immediately to the right of the colon is the number of double bonds, and the number after the hyphen indicates the position of the first double bond from the methyl end.

stored on ice. Ripe females ($n = 29$) were stripped of eggs, weighed, and measured. Age was estimated by the annual ring count of sagittal otoliths as read by two independent readers (courtesy of Dan Makauskas, IDNR). Eggs were placed in dry plastic containers and transported indirectly on ice. A subsample of eggs (5 g) from each female was placed in a sealed bag and immediately frozen between slabs of dry ice and stored at −80°C until analyzed (e.g., for lipids, fatty acids, and thiamine). Unfertilized eggs and sperm were transported to the Illinois Natural History Survey laboratory. Upon arrival, sperm from individual males was checked for motility. Motile semen of 5–6 males was pooled and used for the fertilization of all eggs. Eggs from each female (~100 eggs) were fertilized in triplicate with 100 μL of pooled sperm using the dry method (10 mL of lake water was added to activate the gametes). Fertilized eggs were incubated until hatching in plastic baskets with screen bottoms in California-type hatching trays (Flex-a-Lite Consolidated, Inc., Tacoma, Washington) supplied with flow-through Lake Michigan water at the rate of 5 L/min. Temperature was monitored several times a day and daily mortality was recorded.

When all eggs had hatched, mortality up to hatching (prehatch) was quantified for each female as a mean of three replicates. After hatching, the embryos from each female were sorted for signs of subcutaneous yolk edema and blue sac disease. The mean value of posthatch mortality was calculated for the progeny of each female. For the EMS monitoring part of the experiment (swim-up mortality), we selected 100 individuals with no obvious physical abnormalities from each female and placed them in separate aquaria (30 L). Each aquarium was continuously supplied with ambient-temperature (4–5°C) lake water and continuous aeration. During embryonic and posthatch development, relative age was determined as cumulative degree-days (CDD), which is the product of the mean daily temperature (°C) and the number of days since fertilization. Temperature was measured three times a day, and fish were monitored for any signs of EMS (lethargy, loss of equilibrium, or spiral and erratic swimming). Individuals with signs of EMS were removed from the aquaria and counted. The experiment was terminated at 1,000 CDD.

Lipid and fatty acid analysis.—Total lipids were extracted from unfertilized lake trout eggs with chloroform–methanol (2:1 by volume) containing 0.01% butylated hydroxytoluene as an antioxidant (Folch et al. 1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content determined gravimetrically. September-pack silica cartridges (Waters Corp., Milford, Massachusetts) were used to separate neutral lipids (NL) from phospholipids (PL). Chloroform and methanol were used as mobile phases for NL and PL, respectively (Juaneda and Rocquelin 1985). Fatty acid methyl esters (FAMEs) were prepared following the methods of Metcalfe and Schmitz (1961), separated, and quantified by gas chromatography; the chromatographer (Varian 3900 GC; Varian, Inc., Walnut Creek, California) was equipped with a flame ionization detector, Varian Chrompack capillary column (wall-coated open tubular fused silica 100 m × 0.25 mm coating CPSIL 88 for FAME, film thickness = 0.2), and an auto-injector (CP-8410 AutoInjector; Varian). Helium was used as the carrier gas at a flow of 30 mL/min. The injector and detector temperatures were 270° and 300°C, respectively. The temperature of the oven was held at 175°C for 26 min, then increased to 205°C by increments of 2°C/min and held at 205°C for 24 min. Before transmethylation, a known amount of nonadecanoate acid (19:0; Nu Check Prep, Inc., Elysian, Minnesota) proportional to the amount of NL or PL detected (8 mg per 50 mg of lipids) was added as an internal standard. The individual fatty acid methyl esters were identified by comparing their retention times with those of authentic standard mixtures (FAME mix 37 components; Supleco, Bellefonte, Pennsylvania) and quantified by comparing their peak areas with that of the internal standard. The results for individual fatty acids were expressed as percentages of total identified FAMEs.

Thiamine analysis.—Free thiamine (TH) and its derivatives, thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) were extracted from lake trout eggs according to Brown et al. (1998b). They were then quantified using a high-performance liquid chromatograph (HPLC) system as described by Brown et al. (1998b) and Mancinelli et al. (2003), with slight modifications. The HPLC system consisted of a delivery system pump (Model 506A; Beckman Instruments, Inc., San Ramon, California) equipped with a 20-μL injection loop connected to a 4.6-mm × 150-mm NH (aminopropyl-bonded silica gels, 5-μm bead size; Showa Denko, Tokyo, Japan) Shodex column coupled with an NH_2-packed guard column. The fluorescence detector (BAS, LC22C) was set at 375 nm for excitation and at 430 nm for emission. The mobile phase was composed of potassium phosphate buffer (pH 7.5; 85 mM) plus acetonitrile (65:35). The flow rate was 0.5 mL/min. The column thermostat was set at 30°C. Standard curves for TH, TMP, and TPP were determined using 1 mM of each standard stock solution in 0.01 M HCl. Each standard concentration ranged from 1.0 to 100 nmol/L for linearity. The extraction recovery rates were 94.7 ± 3.0% ($n = 4$) for TH and

over 100% for both TMP and TPP. To determine extraction efficiency, known amounts of each TH, TMP, and TPP standard were added to running samples at the beginning of the extraction, followed by the extraction procedure as described above.

Statistical analysis.—All data were expressed as means ± SDs. Statistical analyses were performed with the Statistical Analysis System (SAS Institute, Inc., Cary, North Carolina). All percentages were arcsine transformed prior to analysis. All data were tested for normality with the Shapiro–Wilk test and for homogeneity of variance with Bartlett's test. The concentrations of each fatty acid were compared between the neutral and phospholipid fractions using one-way analysis of variance (ANOVA). When the assumptions about normality and homogeneity could not be satisfied, a nonparametric method (Kruskal–Wallis test) was used. Stepwise multiple-regression analysis was used to explore the relationships between mortality at a given life stage and individual fatty acids and thiamine vitamers. Linear regression analysis was used to evaluate the relationships between the following variables: (1) free thiamine concentration and mortality (prehatch, posthatch, and swim-up); (2) total thiamine concentration and TH, TMP, and TPP; (3) total thiamine concentration and fatty acid concentrations; and (4) fatty acid concentrations and mortality (prehatch, posthatch, and swim-up).

Results

Twenty-nine mature females (total length, 752 ± 80 mm) were spawned during the fall of 2003. Neither female size nor age was related to any of the biochemical traits measured in the eggs (all $P > 0.05$).

Lipids, Fatty Acids, and Thiamine

Lake trout eggs contained 36 ± 2.5% dry matter and 9.3 ± 0.4% total lipids on a wet-mass basis. The total lipids were equivalent to 26.0 ± 2.3% of the egg dry mass. Neutral lipids comprised 53.7 ± 2.5% of the total lipids, phospholipids 46.3 ± 2.5%. The fatty acid profiles of the eggs were significantly different between these two lipid fractions (Table 1). Saturated fatty acids were significantly higher in the phospholipid fraction than in the neutral lipid fraction, with increased levels of palmitic acid (16:0) and stearic acid (18:0). Neutral lipids were characterized by high levels of monounsaturated fatty acids, mainly palmitoleic acid (16:1[n-7]), oleic acid (18:1[n-9]), and vaccenic acid (18:1[n-7]). Although the concentrations of PUFAs were high in both lipid fractions, they differed significantly, mainly because of a higher level of DHA in the phospholipids. Consequently, the DHA:EPA ratio was two-fold higher in the phospholipid fraction than in the neutral lipid fraction. The lower concentration of AA in the neutral lipids than in the phospholipids resulted in a higher EPA:AA ratio in the neutral lipids.

TABLE 1.—Fatty acid concentration (expressed as a percentage of the total fatty acids detected) in lake trout eggs collected from southwestern Lake Michigan in 2003 (nd = not detected). Analysis of variance revealed significant differences in all fatty acids between the two lipid fractions ($P < 0.001$).

Fatty acid[a]	Neutral lipids	Phospholipids
Saturated		
12:0	0.02 ± 0.01	nd
14:0	2.22 ± 0.15	1.01 ± 0.08
15:0	0.23 ± 0.03	0.25 ± 0.03
16:0	8.11 ± 0.82	12.60 ± 0.53
17:0	0.14 ± 0.03	0.27 ± 0.04
18:0	1.89 ± 0.26	6.74 ± 0.45
All saturated	12.61 ± 1.03	20.87 ± 0.72
Monounsaturated		
14:1	0.07 ± 0.01	nd
16:1(n-9)	1.15 ± 0.22	0.79 ± 0.15
16:1(n-7)	10.92 ± 2.23	2.66 ± 0.62
17:1	0.48 ± 0.07	0.19 ± 0.02
18:1(n-9)	22.73 ± 1.98	9.72 ± 0.58
18:1(n-7)	8.31 ± 1.03	7.82 ± 1.23
20:1(n-9)	1.16 ± 0.19	2.35 ± 0.39
22:1(n-9)	0.07 ± 0.02	nd
All monounsaturated	44.89 ± 3.61	23.54 ± 1.51
Polyunsaturated		
18:2(n-6)	3.39 ± 0.42	0.88 ± 0.12
20:2(n-6)	0.66 ± 0.11	0.99 ± 0.16
20:3(n-6)	0.57 ± 0.06	0.28 ± 0.06
20:4(n-6)	3.97 ± 0.61	7.75 ± 0.91
22:4(n-6)	0.64 ± 0.12	0.49 ± 0.12
22:5(n-6)	0.91 ± 0.23	1.35 ± 0.34
18:3(n-3)	3.03 ± 0.55	0.51 ± 0.10
18:4(n-3)	0.75 ± 0.18	0.10 ± 0.03
20:3(n-3)	0.77 ± 0.18	0.65 ± 0.16
20:4(n-3)	2.48 ± 0.39	0.68 ± 0.11
20:5(n-3)	7.59 ± 1.45	8.14 ± 1.60
22:5(n-3)	5.72 ± 0.57	6.95 ± 1.34
22:6(n-3)	12.02 ± 1.50	26.82 ± 1.32
All polyunsaturated	42.49 ± 3.22	55.59 ± 1.14
All n-6	10.13 ± 1.27	11.74 ± 1.59
All n-3	32.36 ± 2.29	43.85 ± 1.10
DHA:EPA ratio	1.64 ± 0.35	3.42 ± 0.69
EPA:AA ratio	1.96 ± 0.51	1.07 ± 0.29

[a] In the terms in this column, the number to the left of the colon is the number of carbon atoms in the compound, the number immediately to the right of the colon is the number of double bonds, and the number after the hyphen indicates the position of the first double bond from the methyl end. The abbreviations are as follows: DHA = docosahexaenoic acid (22:6[n-3]), EPA = eicosapentaenoic acid (20:5[n-3]), and AA = arachidonic acid (20:4[n-6]).

The free-thiamine concentration in lake trout eggs varied by an order of magnitude among females (0.28 ± 0.04 to 3.83 ± 0.61 nmol/g; Figure 1). Free, monophosphate and pyrophosphate thiamine accounted for 75.8 ± 6.6, 10.4 ± 4.4, and 13.8 ± 4.6%, respectively. The proportions of the three forms of thiamine varied unequally, depending on the amount of total thiamine (the sum of three forms) detected in the eggs. The percent of free thiamine decreased ($r^2 = 0.22$,

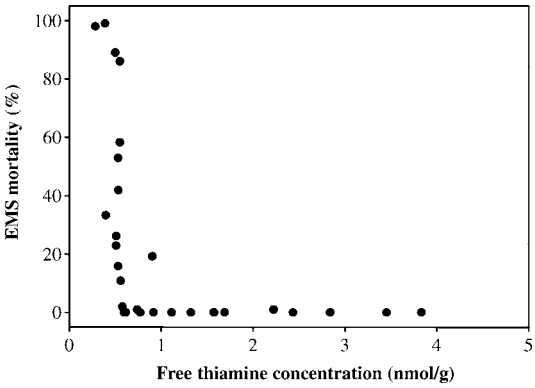

FIGURE 1.—Relationship between the amount of free thiamine in unfertilized lake trout eggs collected from southwestern Lake Michigan in November 2003 and the incidence of early mortality syndrome expressed as percentage of mortality. Each point represents an individual family group.

$P < 0.01$) and that of thiamine pyrophosphate increased ($r^2 = 0.47$, $P < 0.01$) with decreasing total thiamine, whereas the proportion of thiamine monophosphate did not differ (Figure 2). The total thiamine concentration was negatively correlated with the DHA:EPA ratio and positively correlated with the EPA:AA ratio in both the phospholipids ($r^2 = 0.70$, $P < 0.01$; $r^2 = 0.82$, $P < 0.01$) and neutral lipids ($r^2 = 0.62$, $P < 0.01$; $r^2 = 0.57$, $P < 0.01$) of unfertilized eggs (Figure 3).

Mortality dynamics.—A summary of the three observed mortalities (prehatch, posthatch, and swim-up) is presented in Figure 4. Prehatch mortality accounted for 17% of all mortalities across lake trout family groups. Posthatch (blue sac/yolk edema) mortality was relatively infrequent and accounted for an additional 4.5% across family groups. An additional 23% was attributed to swim-up (EMS) mortality. The results of the stepwise multiple-regression analysis performed to identify significant effects of lipid content, individual fatty acid concentrations, and thiamine content of the eggs on the observed mortalities are summarized in Table 2. Regardless of the lipid fraction, cis-7-hexadecenoic acid (16:1[n-9]) was independently correlated with the prehatch mortality. Docosahexaenoic acid in neutral lipids was negatively correlated with posthatch mortality (yolk edema/blue sac disease), whereas docosapentaenoic acid (22:5[n-3]) in phospholipids correlated positively with EMS (swim-up) mortality. Lastly, three predictors were correlated with EMS mortality. When fatty acids from the neutral lipid fraction were entered into the regression model, palmitoleic acid (16:1[n-7]), TPP, and total lipids correlated with EMS mortality ($r^2 = 0.573$), and when fatty acids from the phospholipid

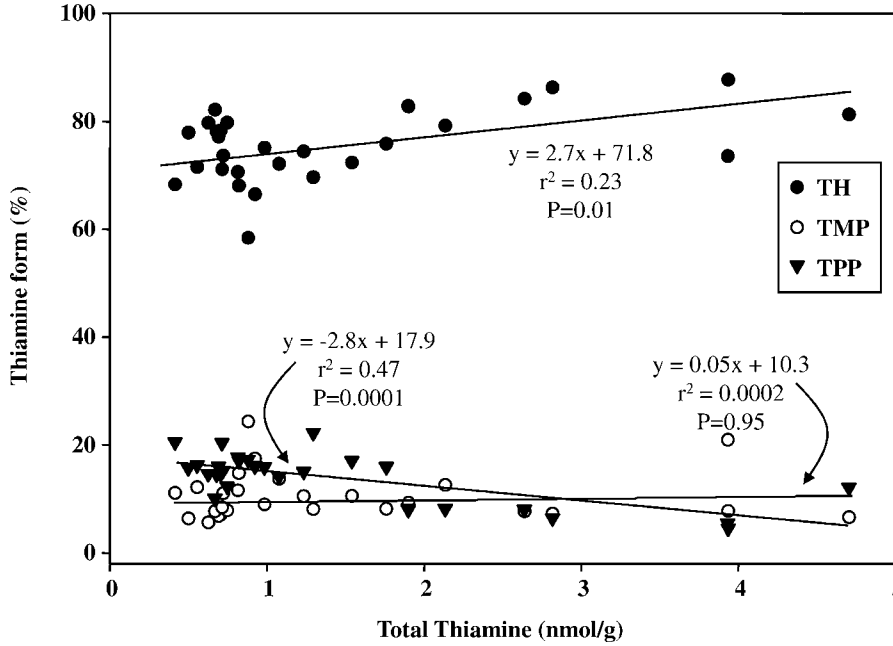

FIGURE 2.—Relationships between total thiamine level and percentage of three forms of thiamine in lake trout eggs collected from southwestern Lake Michigan in November 2003. Abbreviations are as follows: TH = free thiamine, TMP = thiamine monophosphate, and TPP = thiamine pyrophosphate.

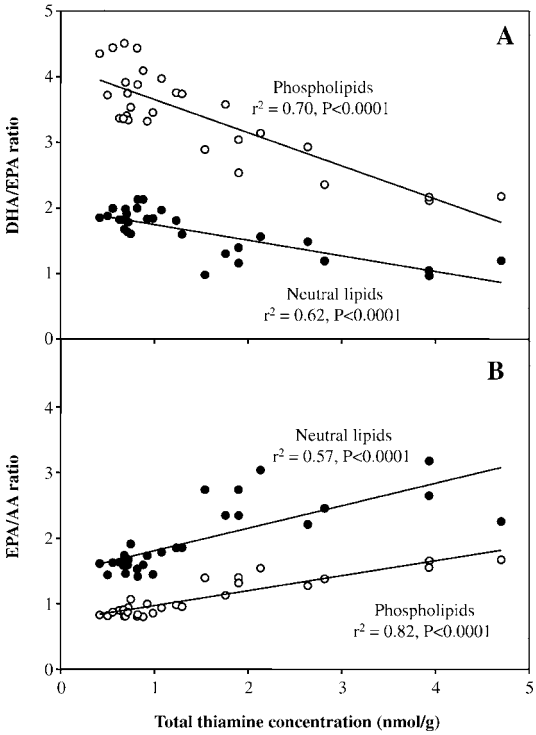

FIGURE 3.—Relationships between total thiamine level and **(A)** the DHA:EPA ratio and **(B)** the EPA:AA ratio in neutral lipids and phospholipids of lake trout eggs collected from southwestern Lake Michigan in 2003.

fraction were entered, linoleic acid (18:2[n-6]), palmitic acid, and TPP were correlated with EMS mortality ($r^2 = 0.596$).

Posthatch mortality attributed to EMS occurred between 600 and 900 CDD and was correlated with the free-thiamine concentration in unfertilized eggs ($r^2 = 0.43$, $P = 0.001$; Figure 5). The offspring of families that incur the highest proportion of EMS-related deaths begin to show EMS symptoms and die much earlier than the offspring of families with lower final EMS mortality. The frequency of EMS mortality was high only in offspring originated from eggs containing low levels (<1 nmol/g) of free thiamine, pointing to this concentration as a critical threshold below which EMS occurs. Similar threshold-type relationships were found between EMS mortality and the DHA:EPA and EPA:AA ratios in both the neutral and phospholipid fractions of egg lipids (Figure 6). Mortality from EMS occurred only when the DHA:EPA ratio was higher than 1.5 in neutral lipids and 3 in phospholipids, while it occurred only at lower EPA:AA ratios in both fractions of egg lipids.

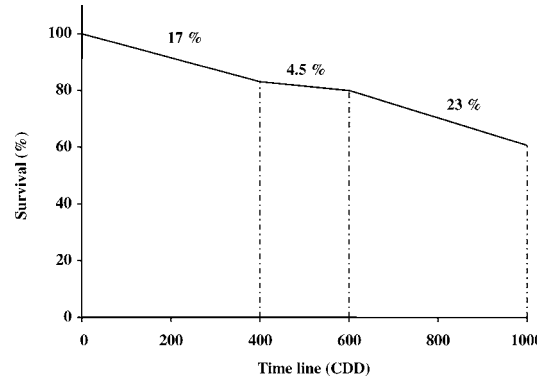

FIGURE 4.—Summary of mortality dynamics across 29 family groups of lake trout offspring from fertilization through the advanced swim-up stage. Prehatch mortality occurred between 0 and 400 cumulative degree-days (CDD) after fertilization, posthatch mortality between 401 and 600 CDD, and swim-up mortality between 601 and 1,000 CDD.

Discussion

Seventeen of the 29 females sampled had initial egg free-thiamine levels below 0.8 nmol/g, the threshold below which an increased frequency of EMS was reported in lake trout from Lake Ontario (Brown et al. 1998a). In our study, the occurrence of EMS among the offspring of females with initial free-thiamine levels below 0.8 nmol/g in eggs was variable and negatively related to thiamine concentration. However, when we employed multiple-regression analysis to test the effects of three forms of thiamine along with fatty acids on the frequency of EMS, we found that TPP was one of the predictors of EMS but that its effect was positive, implying that higher TPP levels would increase EMS frequency in offspring. Although TPP is the metabolically active form in most animals, TH is the predominant form of thiamine in lake trout eggs. In thiamine-deficient eggs the proportion of TH decreases in favor of TPP (see Figure 2). As reported by Honeyfield et al. (2007), lake trout do not initially require high levels of TPP because they undergo a long embryonic development (2–4 months) prior to hatching. The low levels of TH in the eggs will affect the production of TPP later on during embryonic development and consequently the level of TPP required for the normal development of lake trout embryos may not be attained.

The signs often observed in individuals affected by EMS, such as loss of equilibrium, hyperexcitability, and erratic and spiral swimming, have obvious neurological underpinnings, whereas growth impairment leading to lethargy and anorexia implicates disruptions of energy metabolism. The amount of

TABLE 2.—Results of the stepwise multiple-regression analysis of lake trout mortality. Prehatch, posthatch, and swim-up mortalities were used as dependent variables, successively. The following independent variables were considered: total lipids (TL), free thiamine (TH), thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and all of the fatty acids from either the neutral lipid fraction or the phospholipid fraction.

Dependent variable	Predictor[a]	Standardized regression coefficient(s)	*F*-value	*P*-value	Change in r^2 (%)
Including neutral lipids					
Prehatch mortality	16:1(n-9)	0.421	5.82	0.023	17.7
Posthatch mortality	22:6(n-3)	−0.417	5.68	0.024	17.4
Swim-up mortality	16:1(n-7)	0.528	19.10	<0.001	41.4
	TPP	0.301	12.94	<0.001	8.4
	TL	0.272	11.17	<0.001	7.4
Including phospholipids					
Prehatch mortality	16:1(n-9)	0.508	9.38	0.005	25.8
Posthatch mortality	22:5(n-3)	0.457	7.13	0.013	20.9
Swim-up mortality	18:2(n-6)	−0.404	19.29	<0.001	41.7
	16:0	−0.342	14.28	<0.001	10.7
	TPP	0.294	12.28	<0.001	7.2

[a] See Table 1 for an explanation of terms such as 16:1(n-9).

thiamine present in the early development of lake trout is, without question, a key factor that has been previously linked to the onset of EMS (Brown et al. 2005b). It is imperative, however, to discuss the role of thiamine in a broader context, one that would permit us to better understand the mechanism or mechanisms ultimately responsible for this disease.

An inadequate level of thiamine in the diet depresses the activities of three enzymes (pyruvate decarboxylase, alpha ketoglutarate decarboxylase, and transketolase) that require thiamine pyrophosphate as a cofactor (Gubler 1991). It has been demonstrated that fatty acid synthesis in thiamine-deficient cultured cells was reduced to 15% of the rate in thiamine-supplemented cells (Volpe and Marasa 1978). This decrease was accompanied by a similar decrease in the activity of acetyl-CoA carboxylase and fatty acid synthetase, the two enzymes sequentially involved in fatty acid synthesis. The importance of thiamine as a regulator of fatty acid synthesis becomes obvious when we consider the rapid development of the nervous system in lake trout embryos and the high demand for PUFAs as structural components of neural biomembranes.

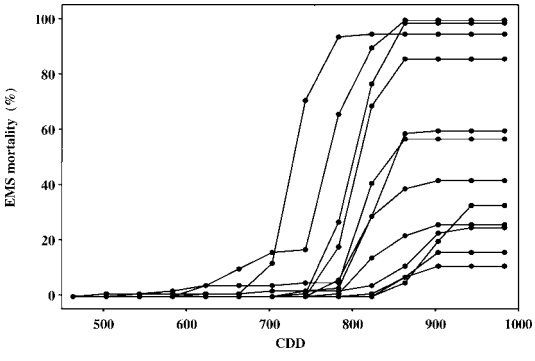

FIGURE 5.—Dynamics of EMS mortality in progeny of 12 lake trout family groups. For clarity, only families with at least 10% EMS mortality are presented.

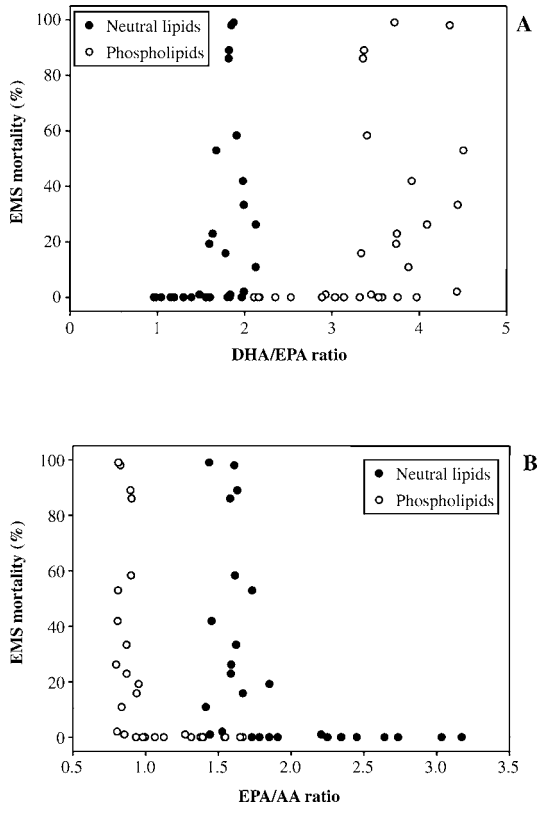

FIGURE 6.—Relationship between EMS mortality and **(A)** the DHA:EPA ratio and **(B)** the EPA:AA ratio from both the neutral lipid and phospholipid fractions of lake trout eggs.

Biomembranes require an adequate degree of fluidity to function properly at low temperatures, and high PUFA content in these structures provides such fluidity. The prolonged period (November through March) of lake trout embryonic development takes place at low temperatures (~1–2°C; Mac et al. 1985). At these thermal conditions, low thiamine levels or thiamine deficiency may retard fatty acid elongation and desaturation and lead to dysfunctional neural biomembranes that will eventually be manifested through clinical symptoms of EMS.

Thiamine and fatty acids, along with all other nutrients, are deposited in lake trout eggs during vitellogenesis, a process of ovarian maturation that takes several months and is influenced (quantitatively and qualitatively) by the availability of these nutrients in the diet and ultimately the food chain. With respect to fatty acids, however, this process does not follow the simple rule "more in the diet, more in the eggs," as certain fatty acids (or more generally, fatty acid families, e.g., n-3 and n-6) will have differential or competitive effects with respect to their rate of deposition in eggs. Furuita et al. (2002), for example, demonstrated that the EPA content of eggs is more sensitive to changes in dietary n-3 PUFAs than to DHA, while an increased level of EPA in eggs depresses AA deposition. In general, however, the fatty acid profiles of fish eggs are often characterized by high levels of n-3 PUFAs, especially DHA, EPA, monounsaturated fatty acid (MUFA) (18:1[n-9]), and saturated fatty acids (SAFA), with apparent selective deposition of essential n-3 fatty acids, particularly DHA. The fatty acid profiles of the lake trout eggs in this study follow this general pattern.

A DHA:EPA ratio of 2:1 is commonly found in the egg phospholipids of fish (Sargent et al. 1995). In our study, the average DHA:EPA ratio in phospholipids was 3.4 (range, 2.1–4.5), and the relative balance of these two key PUFAs was negatively correlated with the total thiamine level. The concomitantly high level of DHA and/or low level of EPA, along with a low level of thiamine, resulted in the above relationship in lake trout eggs and led us to believe that the variable proportion of alewives in the diet was a contributor. Not only are alewives known to be a poor source of thiamine owing to their high thiaminase activity (Fitzsimons et al. 2005), their fatty acid profiles are characterized by relatively high DHA and relatively low EPA levels (Czesny et al., unpublished). Consequently, a high proportion of alewives in the lake trout diet would result in a negative relationship between the DHA:EPA ratio and the thiamine concentration of their eggs. A similar observation of higher DHA content and lower thiamine in M74-affected eggs in Atlantic salmon was reported by Pickova et al. (1998) and interpreted as a potential imbalance between oxidative potential (e.g., elevated DHA) and antioxidant capacity (e.g., lower astaxanthin). Earlier, reduced levels of antioxidant agents in M74-affected Atlantic salmon were suggested as a reason for increased lipid peroxidation (Pettersson and Lignell 1996). Brown et al. (2005a) reported higher DHA levels in the eggs of EMS-negative groups of Chinook salmon from the Swan River (Lake Huron). However, in the same study, no such pattern was noted for either Chinook salmon from the Little Manistee River (Lake Michigan) or coho salmon from two Lake Michigan stocks (the Platte River and Thompson Creek). In our case, foraging on alewives that contain high DHA levels seems a more direct and plausible cause for the elevated concentration of DHA in lake trout eggs. Although the combination of low thiamine, high DHA, and low EPA in lake trout eggs can easily be associated with alewives as a main forage species, the interrelationships among thiamine, DHA, EPA, AA, and mortality at the different early life stages deserve further investigation.

During fish development, all fatty acids are catabolized to some extent, but some are preferentially conserved for incorporation into structural lipids (Ronnestad et al. 1995; Wiegand 1996; Tocher 2003). Oleic acid plays important structural roles in membranes, and its insertion into the sn-1 position of membrane phospholipids contributes largely to homeoviscous adaptations (Dey et al. 1993; Buda et al. 1994). Palmitoleic acid (16:1[n-7]) is used primarily for catabolism and does not have a predominant role in structural lipids (Bell and Tocher 1989; Bell and Dick 1991; Kikuchi et al. 1999). This indicates the advantage of oleic acid deposition to the egg reserves to ensure proper membrane development; no such advantage exists for palmitoleic acid deposition. Interestingly, in our study palmitoleic acid in egg neutral lipids correlated positively with EMS mortality whereas linoleic acid (18:2[n-6]) from egg phospholipids correlated negatively (Table 2). Although these relationships do not allow us to draw any cause–effect conclusions, they clearly indicate a need for controlled experiments to provide additional insights into the physiological mechanisms that lead to EMS.

Both AA and EPA serve as precursors for families of chemical messengers collectively known as eicosanoids. Arachidonic acid is the preferred substrate for the two eicosanoid synthesis systems, and eicosanoids derived from AA are generally more biologically active than those derived from EPA (Tocher 2003). The importance of an adequate level of AA and an appropriate EPA:AA ratio have been recognized for

both freshwater and marine species. In particular, elevated levels of AA have been associated with superior resistance to infection, better egg quality, and growth in salmonids (Ackman and Takeuchi 1986; Bell and Sargent 2003). By contrast, abnormalities have also been associated with low EPA:AA ratios (Sargent et al. 1995). Thus, it appears that optimal EPA:AA ratios are very important for proper development. The significance of an optimal EPA:AA ratio was also apparent in this study and associated with an increased frequency of EMS when it fell below 2 in neutral lipids and below 1 in phospholipids (Figure 6). We also noted a similar threshold-like response of EMS frequency to a variable DHA:EPA ratio in the neutral and phospholipids of lake trout eggs. Both of these relationships—egg EPA:AA ratio versus EMS mortality and egg DHA:EPA ratio versus EMS mortality—along with the strong connection between thiamine and EMS mortality, are driven by the nutritional quality of adult female lake trout forage. Thus, differences in prey assemblages related to either temporal or special fluctuations may be critical in the onset of EMS and other developmental dysfunctions linked to maternal nutrition.

Acknowledgments

We thank Steve Robillard and Dan Makauskas of the Illinois Department of Natural Resources for their help with obtaining lake trout gametes for this study. Special thanks are extended to Robert Goralczyk for his technical assistance during egg collection and incubation. We also thank the Illinois Natural History Survey for financial support through the Seed Grant initiative.

References

Ackman, R. G., and T. Takeuchi. 1986. Comparison of fatty acids and lipids of smolting hatchery-fed and wild Atlantic salmon *Salmo salar*. Lipids 21:117–120.

Adams, S. M. 1998. Ecological role of lipids in the health and success of fish reproduction. Pages 132–160 *in* M. T. Arts and B. C. Wainman, editors. Lipids in freshwater ecosystems. Springer-Verlag, New York.

Amcoff, P., H. Borjeson, J. Lindeberg, and L. Norrgren. 1998. Thiamine concentrations in feral Baltic salmon exhibiting the M74 syndrome. Pages 82–89 *in* G. McDonald, J. D. Fitzsimons, and D. C. Honeyfield, editors. Early life stage mortality syndrome in fishes of the Great Lakes and Baltic Sea. American Fisheries Society, Symposium 21, Bethesda, Maryland.

Bell, J. G., and J. R. Dick. 1991. Molecular species composition of the major diacyl glycerophospholipids from muscle, liver, retina, and brain of cod (*Gadus morhua*). Lipids 26:565–573.

Bell, J. G., and J. R. Sargent. 2003. Arachidonic acid in aquaculture feeds: current status and future opportunities. Aquaculture 218:491–499.

Bell, M. V., and D. R. Tocher. 1989. Molecular species composition of the major phospholipids in brain and retina from rainbow trout (*Salmo gairdneri*): occurrence of high levels of di-(n-3) polyunsaturated fatty acid species. Biochemistry Journal 264:909–915.

Bronte, C. R., C. C. Krueger, M. E. Holey, M. L. Toneys, R. L. Eshenroder, and J. L. Jonas. 2008. A guide for the rehabilitation of lake trout in Lake Michigan. Great Lakes Fishery Commission, Miscellaneous Publication 2008-01, Ann Arbor, Michigan.

Brown, S. B., M. T. Arts, L. R. Brown, M. Brown, K. Moore, M. Villella, J. D. Fitzsimons, D. C. Honeyfield, D. E. Tillitt, J. L. Zajicek, M. Wolgamood, and J. G. Hnath. 2005a. Can diet-dependent factors help explain fish-to-fish variation in thiamine-dependent early mortality syndrome? Journal of Aquatic Animal Health 17:36–47.

Brown, S. B., J. D. Fitzsimons, D. C. Honeyfield, and D. E. Tillitt. 2005b. Implications of thiamine deficiency in Great Lakes salmonines. Journal of Aquatic Animal Health 17:113–124.

Brown, S. B., J. D. Fitzsimons, V. P. Palace, and L. Vandenbyllaardt. 1998a. Thiamine and early mortality syndrome in lake trout. Pages 18–25 *in* G. McDonald, J. D. Fitzsimons, and D. C. Honeyfield, editors. Early life stage mortality syndrome in fishes of the Great Lakes and the Baltic Sea. American Fisheries Society, Symposium 21, Bethesda, Maryland.

Brown, S. B., D. H. Honeyfield, and L. Vandenbyllaardt. 1998b. Thiamine analysis in fish tissues. Pages 73–81 *in* G. McDonald, J. D. Fitzsimons, and D. C. Honeyfield, editors. Early life stage mortality syndrome in fishes of the Great Lakes and the Baltic Sea. American Fisheries Society, Symposium 21, Bethesda, Maryland.

Buda, C., I. Dey, N. Balogh, L. I. Horvath, K. Maderpach, M. Juhasz, Y. K. Yeo, and T. Farkas. 1994. Structural order of membranes and composition of phospholipids in fish brain cells during thermal acclimatization. Proceedings of the National Academy of Sciences of the USA 91:8234–8238.

Cantrell, S. M., J. Joy-Schlezinger, J. J. Stegeman, D. E. Tillitt, and M. Hannink. 1998. Correlation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced apoptotic cell death in the embryonic vasculature with embryotoxicity. Toxicology and Applied Pharmacology 148:24–34.

Czesny, S., S. Kolkovski, K. Dabrowski, and D. Culver. 1999. Growth, survival, and quality of juvenile walleye *Stizostedion vitreum* as influenced by n-3 HUFA enriched *Artemia* nauplii. Aquaculture 178:103–115.

Dey, I., C. Buda, T. Wiik, J. E. Halver, and T. Farkas. 1993. Molecular and structural composition of phospholipid membranes in livers of marine and freshwater fish in relation to temperature. Proceedings of the National Academy of Sciences of the USA 90:7498–7502.

Eschmeyer, P. H. 1957. The near extinction of lake trout in Lake Michigan. Transactions of the American Fisheries Society 85:102–119.

Fernandez-Palacios, H., M. S. Izquierdo, L. Robaina, A. Valencia, M. Salhi, and J. M. Vergara. 1995. Effect of n-3 HUFA level in broodstock diets on egg quality of

gilthead sea bream (*Sparus aurata* L.). Aquaculture 132:325–337.

Fisher, J. P., J. M. Spitsbergen, and R. Getchell. 1995. Reproductive failure in landlocked Atlantic salmon from New York Finger Lakes (1): investigation into the etiology and epidemiology of the "Cayuga syndrome." Journal of Aquatic Animal Health 7:81–94.

Fitzsimons, J. D., B. Williston, J. L. Zajicek, D. E. Tillitt, S. B. Brown, L. R. Brown, D. C. Honeyfield, D. M. Warner, L. G. Rudstam, and W. Pearsall. 2005. Thiamine content and thiaminase activity of ten freshwater stocks and one marine stock of alewives. Journal of Aquatic Animal Health 17:26–35.

Furuita, H., H. Tanaka, T. Yamamoto, N. Suzuki, and T. Takeuchi. 2002. Effect of high levels of n-3 HUFA in broodstock diet on egg quality and egg fatty acid composition of the Japanese flounder *Paralichthys olivaceus*. Aquaculture 210:323–333.

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biology and Chemistry 226:497–509.

Gubler, C. J. 1991. Thiamin. Pages 233–281 *in* L. J. Machlin, editor. Handbook of vitamins, 2nd edition. Marcel Dekker, New York.

Hazel, J. R. 1989. Cold adaptation in ectotherms: regulation of membrane function and cellular metabolism. Pages 1–50 *in* L. C. H. Wang, editor. Advances in comparative and environmental physiology. Springer-Verlag, Berlin.

Hile, R., P. H. Eschmeyer, and G. F. Lunger. 1951. Decline of the lake trout fishery in Lake Michigan. U.S. Fish and Wildlife Service Fishery Bulletin 52:77–95.

Honeyfield, D. C., C. S. Vangergoot, P. W. Bettoli, J. P. Hinterkopf, and J. L. Zajicek. 2007. Thiamine and fatty acid content of walleye tissue from three southern U.S. reservoirs. Journal of Aquatic Animal Health 19:84–93.

Juaneda, P., and G. Rocquelin. 1985. Rapid and convenient separation of phospholipids and nonphosphorus lipids from rat heart using silica cartridges. Lipids 20:40–41.

Kikuchi, K., K. Sakai, T. Suzuki, and K. Tanaka. 1999. Fatty acid composition of choline and ethanolamine glycerophospholipid subclasses in heart tissue of mammals and migratory demersal fish. Comparative Biochemistry and Physiology 124B:1–6.

Mac, M. J., C. C. Edsall, and J. G. Seelye. 1985. Survival of lake trout eggs and fry reared in water from the upper Great Lakes. Journal of Great Lakes Research 11(4):520–529.

Mancinelli, R., M. Ceccanti, M. S. Guiducci, G. F. Sasso, G. Sebastiani, M. L. Attilia, and J. P. Allen. 2003. Simultaneous liquid chromatographic assessment of thiamine, thiamine monophosphate, and thiamine diphosphate in human erythrocytes: a study on alcoholics. Journal of Chromatography B 789:355–363.

Madenjian, C. P., T. J. DeSorcie, and R. M. Stedman. 1998. Ontogenic and spatial patterns in diet and growth of lake trout in Lake Michigan. Transactions of the American Fisheries Society 127:236–252.

Marcquenski, S. V., and S. B. Brown. 1997. Early mortality syndrome in the Great Lakes. Pages 135–152 *in* R. M. Rolland, M. Gilbertson, and R. E. Peterson, editors. Chemically induced alterations in functional development and reproduction in fishes. Society of Environmental Toxicology and Chemistry, Pensacola, Florida.

Marshall, C. T., N. A. Yaragina, Y. Lambert, and O. S. Kjesbu. 1999. Total lipid energy as a proxy for total egg production by fish stocks. Nature (London) 402:288–290.

Metcalfe, L. D., and A. A. Schmitz. 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. Analytical Chemistry 33:363–364.

Pettersson, A., and A. Lignell. 1996. Decreased astaxanthin levels in the Baltic salmon and the M74 syndrome. Pages 28–29 *in* B.-E. Bengtsson, C. Hill, and S. Nellbring, editors. Proceedings of the 2nd workshop on reproduction disturbances in fish. Swedish Environmental Protection Agency, Report 4534, Stockholm.

Pickova, J., A. Kiessling, A. Pettersson, and P. Dutta. 1998. Comparison of fatty acid composition and astaxanthin content in healthy and by M74 affected salmon eggs from three Swedish river stocks. Comparative Biochemistry and Physiology 120B:265–271.

Ronnestad, I., R. N. Finn, I. Lein, and O. Lie. 1995. Compartmental changes in the contents of total lipid, lipid classes, and their associated fatty acids in developing yolk sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L.). Aquaculture Nutrition 1:119–130.

Sargent, J., G. Bell, M. V. Bell, R. J. Henderson, and D. R. Tocher. 1995. Requirement criteria for essential fatty acids. Journal of Applied Ichthyology 11:183–198.

Sargent, J. R. 1995. Origins and functions of egg lipids: nutritional implications. Pages 353–372 *in* N. R. Bromage and R. J. Roberts, editors. Broodstock management and egg and larval quality. Blackwell Scientific Publications, Cambridge, Massachusetts.

Sargent, J. R., R. J. Henderson, and D. R. Tocher. 1989. The lipids. Pages 153–218 *in* J. Halver, editor. Fish nutrition, 2nd edition. Academic Press, New York.

Tillitt, D. E., J. L. Zajicek, S. B. Brown, L. R. Brown, J. D. Fitzsimons, D. C. Honeyfield, M. E. Holey, and G. M. Wright. 2005. Thiamine and thiaminase status in forage fish of salmonines from Lake Michigan. Journal of Aquatic Animal Health 17:13–25.

Tocher, D. R. 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11:107–184.

Volpe, J. J., and J. C. Marasa. 1978. A role for thiamine in the regulation of fatty acid and cholesterol biosynthesis in cultured cells of neural origin. Journal of Neurochemistry 30:975–981.

Watanabe, T., T. Fujimura, M. J. Lee, K. Fukusho, S. Satoh, and T. Takeuchi. 1984. Effect of nutritional composition of diets on chemical components of red sea bream broodstock and eggs produced. Bulletin of the Japanese Society of Scientific Fisheries 50:503–515.

Watanabe, T., M. Ohta, C. Katajima, and S. Fujita. 1982. Improvements of dietary value of brine shrimp *Artemia salina* for fish larvae by feeding them ω3 highly unsaturated fatty acids. Bulletin of the Japanese Society of Scientific Fisheries 48:1775–1782.

Wiegand, M. D. 1996. Composition, accumulation, and utilization of yolk lipids in teleost fish. Reviews in Fish Biology and Fisheries 6:259–286.