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[Article]

Effects of Vitamin B_1 (Thiamine) Deficiency in Lake Trout Alevins and Preventive Treatments

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Abstract.—The objectives of this study were to examine the effect of thiamine immersion of fish from a population known for compromised survival as a result of early mortality syndrome (EMS) and to investigate the cause–response relationship between thiamine concentration and lesions in tissues in swim-up-stage lake trout *Salvelinus namaycush* alevins. Lake trout eggs from 14 fish from Lake Michigan were artificially fertilized and the progeny divided into two groups based on the thiamine concentration (low [<0.73 nmol/g] or high [>0.85 nmol/g]) in the unfertilized eggs. Progeny were treated or not with a thiamine solution (2,000 mg/L for 2 h) at hatching or the swim-up stage. The survival of progeny in control groups at the swim-up stage correlated with thiamine concentration. The low thiamine-treated groups had significantly higher survival between the swim-up stage (812.0 degree-days) and 16 d after swim-up (963.3 degree-days) than the control groups; the survival of the high thiamine-treated groups did not differ between treated and control fish, regardless of the treatment at hatching and the swim-up stage. Control alevins that had low thiamine levels showed EMS, which resulted in 94.9–100% mortality 16 d after the swim-up stage. No pathological changes were observed in the brain, olfactory lobe, eye, liver, or muscle in alevins of high thiamine-treated group. Glycogen deposits in the liver of alevins from the low control group were variable, no glycogen being observed in the hepatocytes of 7 of the 24 fish. We demonstrate that thiamine treatment at swim-up enhances the survival of EMS-affected lake trout relative to treatment at hatching.

The etiology of early mortality syndrome (EMS) in populations of lake trout *Salvelinus namaycush* in Lake Michigan is considered an example of "environmentally associated disease." Large numbers of hatchery-born lake trout are stocked in Lake Michigan every year. Although these fish generally survive to adulthood and produce viable eggs, no significant natural recruitment has been recorded. Poor lake trout recruitment in the Great Lakes has been linked to nutritional deficiencies, especially inadequate levels of thiamine (vitamin B_1; Fitzsimons 1995; Brown et al. 2005). A similar example of thiamine deficiency (M74) has been described in the Baltic Sea, where low concentrations of thiamine in eggs have resulted in high mortalities at the yolk sac stage for several salmonid species as well as Atlantic cod *Gadus morhua* (Amcoff et al. 1998). Mortality from thiamine deficiency is the result of predator fish consuming prey containing high levels of thiaminase, a thiamine-degrading enzyme that has been found in prey species such as alewife *Alosa pseudoharengus* and rainbow smelt *Osmerus mordax*. Laboratory experiments revealed that 67% of lake trout families showed high incidences of EMS when free-thiamine levels in eggs dropped below 0.8 nmol/g (Brown et al. 1998). Histopathological studies of yolk sac alevins of Atlantic salmon *Salmo salar* with M74 syndrome have also been carried out; however, no direct link to thiamine concentration in the eggs or embryo or alevin tissues has been established (Lundström et al. 1999). The latter authors concluded that the cause of the wide spectrum of microscopic lesions observed in the brain (i.e., cell necrosis, periventricular hydropic degeneration, hyperplasia of capillaries, and hemorrhages) might have some connection to thiamine deficiency despite the absence of direct evidence.

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TABLE 1.—Characteristics of female lake trout and their progeny used in the present study. There were significant differences ($P = 0.013$) in the amount of total thiamine in the unfertilized eggs among the four groups (H-LO, H-HI, S-LO, S-HI; 058 ± 0.11^{y}, 3.46 ± 1.84^{z}, 0.64 ± 0.12^{y}, 2.05 ± 0.85^{yz}, respectively [values without a superscript letter in common are significantly different]) but no differences in mortality at the eyed embryo stage ($P = 0.185$). NM = not measured.

Fish	Origin[a]	Location	Length (mm)	Body mass (g)	Total thiamine (nmol/g of tissue) in unfertilized eggs	Mortality (%) at eyed embryo stage[b]	Group[c]	Alevin body mass (mg/fish)		Number of progeny at hatching
								At swim-up	After fixation	
1	J	South	785	2,500	0.46 ± 0.04	1.5 ± 1.3	H-LO	124.5 ± 19.2	NM	221
2	J	South	765	4,750	0.60 ± 0.04	0.4 ± 0.8		119.1 ± 5.4	NM	144
3	J	South	660	2,300	0.67 ± 0.04	1.6 ± 1.6		75.1 ± 0.8	NM	235
4	J	South	715	3,550	3.74 ± 0.25	1.1 ± 0.5	H-HI	121.6 ± 6.9	NM	270
5	J	South	765	4,350	4.10 ± 0.08	0.4 ± 0.7		107.9 ± 8.0	NM	181
6	W	South	680	2,950	0.85 ± 0.08	0.3 ± 0.6		111.3 ± 6.1	NM	164
7	J	South	670	2,750	5.16 ± 0.25	8.9 ± 2.8		100.6 ± 5.1	NM	96
8	C	North	696	NM	0.66 ± 0.02	3.9 ± 2.3	S-LO	95.0 ± 3.1	70.8 ± 3.9	189
9	C	North	759	NM	0.73 ± 0.04	8.1 ± 2.5		78.1 ± 10.7	59.9 ± 5.4	242
10	C	North	734	NM	0.70 ± 0.07	2.4 ± 2.3		91.7 ± 5.1	64.8 ± 6.1	258
11	C	North	780	NM	0.47 ± 0.02	4.1 ± 1.5		130.2 ± 2.0	95.4 ± 4.5	126
12	C	North	705	NM	3.03 ± 0.33	0.4 ± 0.7	S-HI	100.5 ± 4.1	78.7 ± 4.0	208
13	W	South	790	5,000	1.61 ± 0.08	0.3 ± 0.6		122.2 ± 7.1	93.1 ± 7.4	312
14	W	South	665	2,850	1.51 ± 0.06	2.1 ± 0.9		96.5 ± 5.1	76.7 ± 3.1	182

[a] J = Julian's Reef, W = Waukegan Reef South, and C = Clay Banks Reef.
[b] 100 × number of dead eggs until eyed stage/number of fertilized eggs.
[c] Lake trout treated with or without thiamine at 50% hatching (H) or 50% swim-up (S) with low (LO) or high (HI) thiamine levels in the unfertilized eggs.

The purpose of the present study was to describe the cause–response relationship between the thiamine concentration in yolk-sac-stage embryos and alevins and disease development at the swim-up stage (i.e., lesions in brain, eye, liver, and muscle tissues) for lake trout. It was anticipated that thiamine bath treatments would eliminate mortality in affected fish; however, the association between the severity of pathologies, thiamine concentration, and post-EMS syndrome viability remained to be elucidated.

Methods

Lake trout eggs and alevins.—Adult lake trout were collected in October 2006 from two sites in Lake Michigan, Clay Banks Reef (the northern site) and Waukegan and Julian's reefs (the southern site) (Table 1). The total length (TL) and body mass (BM) of females were measured, and eggs were sampled after ovulation. Eggs were collected before fertilization (unfertilized eggs) for thiamine concentration analysis separately from each female into a dry ice container and kept at −80°C until analysis. Eggs from 14 females were fertilized individually with the sperm of several males and the embryos transported to a laboratory at Ohio State University in Columbus and incubated in a semirecirculating system at 5–9°C.

Thiamine treatment.—One-half of the yolk sac alevins from seven females having high (>0.85 nmol/g [HI]) or low (<0.73 nmol/g [LO]) thiamine levels (based on the total thiamine concentration in the unfertilized eggs; Table 1) were treated with thiamine hydrochloride (T) or not (C) at the 50% hatching stage (H), while one-half of the alevins from another seven females having high or low thiamine levels were treated at the 50% swim-up stage (S) (Figure 1). There were thus eight treatment groups, designated as follows: H-LO-T, H-LO-C, H-HI-T, H-HI-C, S-LO-T, S-LO-C, S-HI-T, and S-HI-C. The thiamine

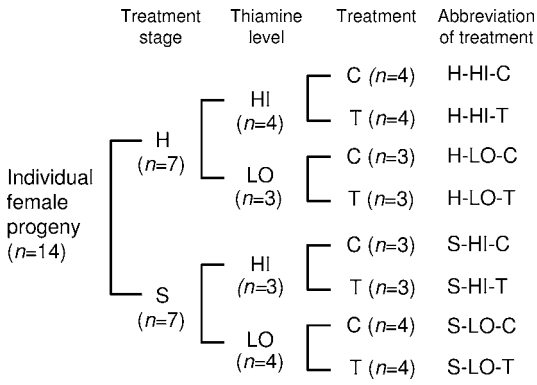

FIGURE 1.—Experimental design for thiamine treatment of Lake Michigan lake trout. The codes H and S indicate the time of treatment with thiamine at 50% hatching (516–569 degree-days) and 50% swim-up stage (775–838 degree-days), respectively. The codes HI and LO indicate two groups of the high (>0.85-nmol/g) or low (<0.73-nmol/g) total thiamine concentrations in the unfertilized eggs. Each replicate of HI and LO fish was split into a control group (C) and a treatment group (T). Sample sizes (*n*) are given in parentheses.

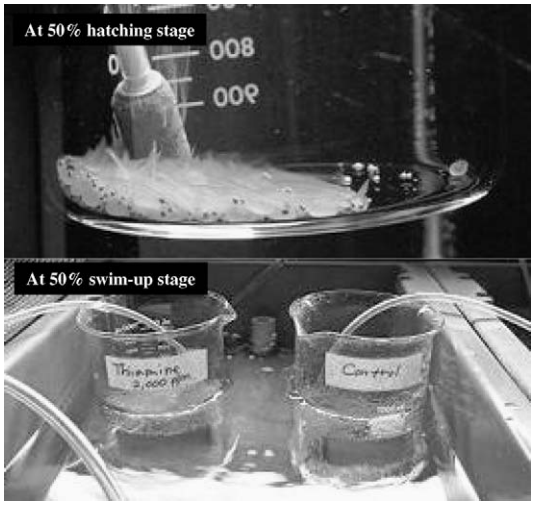

FIGURE 2.—Administration of thiamine treatment to Lake Michigan lake trout at 50% hatching or 50% swim-up stage; the treatments consisted of 2,000 mg/L of buffered thiamine solution (pH 7.0) for 120 min.

treatment solution was prepared according to the method described by Amcoff et al. (1998). One gram of thiamine hydrochloride (Sigma-Aldrich, St. Louis, Missouri) was diluted in 500 mL of system water to achieve a concentration of 2,000 mg/L. The thiamine treatment solution was adjusted to a pH of 7.0 with 2 M NaOH and aerated for 30 min to remove excess CO_2 before fish immersion. Each of the treatment groups was immersed with aeration in the buffered thiamine solution (2,000 mg/L) for 2 h at approximately 6–9°C, while the control groups were immersed in the same volume of system water in the same condition without thiamine (sham treatment; Figure 2). The thiamine treatment solution was collected at 0, 30, 150, and 270 min to monitor possible chemical decomposition of the thiamine. After treatment, all groups were transferred to glass tanks and reared in a semirecirculating system at 8–12°C (3–4 tanks per treatment).

Eyed stage embryos, 50% hatched alevins, and 50% swim-up-stage alevins were sampled to address the question of thiamine utilization during embryogenesis. Alevins treated at the 50% hatching stage with thiamine immersion and control groups of fish were collected for vitamin analysis from 22 to 28 d after treatment at the swim-up stage. Alevins treated at the 50% swim-up stage were collected 1 h after immersion. We rinsed thiamine-treated lake trout alevins with a system water bath three times and kept them for 1 h in the system water to avoid the residual thiamine staying on the body surfaces. Samples were kept at −80°C for thiamine analysis. Alevins from females with low and high levels of unfertilized egg thiamine were used to record symptoms of EMS, as described in Fitzsimons et al. (1995). Fish behavior, such as swimming activity and equilibrium, were recorded prior to treatment and several hours and days after treatment. To account for the effect of EMS on survival, proportional survival was calculated between hatching and the swim-up stage and from swim-up to 16 d after the initiation of feeding. Fish were examined for (1) skeleton deformities, (2) subcutaneous yolk edema, and (3) signs of loss of equilibrium and spiral and erratic swimming.

Fish thermal age.—Degree-days (the mean water temperature [°C] multiplied by the number of days) were calculated based on the temperature and observation days as presented by Allen et al. (2005) for predicting the eyed embryo and hatching stages of lake trout. The average water temperature for individual females from the beginning of egg incubation to the eyed embryo stage ranged from 6.5°C to 8.1°C; thus, the degree-days ranged from 290.3 to 310.3. Fifty-percent hatching was reached at 516–569 degree-days and the swim-up stage at 775–838 degree-days.

Thiamine analysis.—High performance liquid chromatography (HPLC) analysis of free thiamine and its phosphorylated derivatives (thiamine monophosphate and thiamine diphosphate) was slightly modified from Brown et al. (1998). The HPLC system consisted of a delivery system pump (110B; Beckman Instruments) equipped with a 20-μL injection loop connected to a 4.6-mm × 250-mm (Waters Spherisorb; 5 μm NH2, for eggs) or 4.6-mm × 150-mm (Luna; 5 μm NH2, for whole fish body analysis) column coupled with an NH2-packed guard column. The fluorescence detector (FP-920, JASCO Co.) was set at 375 nm for excitation and 430 nm for emission. Standards of thiamine and its phosphorylated forms were prepared. For vitamin analysis, 600 μL of 2% trichloroacetic acid (TCA) extraction solution was added to lake trout samples and the material was gently homogenized for 30 s either by hand or with a low-speed tissue grinder. Water samples from the immersion experiments were analyzed at a desired concentration without an extraction process. The homogenized samples were placed in a boiling water bath for 5–6 min and then cooled on ice for 10 min. After cooling, the samples were supplemented by 600 μL of ice-cold 10% TCA solution and vortexed to mix them. Then the samples were centrifuged at 14,000 × gravity for 15 min at 4°C. The clear supernatants (1 mL) were transferred into glass test tubes (10 mL capacity). To remove the TCA and lipids, the sample extracts in the test tubes were washed with 4 volumes of ethyl acetate-hexane solution (3:2 on a volume basis). The washed sample of 0.5-mL volume was then transferred into an Eppendorf tube and oxidized to

thiochrome by adding 25 μL of 30 mM $K_3Fe(CN)_6$. To increase the pH of the sample extracts, 25 μL of 0.8 M NaOH was added. The oxidized sample extracts were then vortexed and filtered before injection into the HPLC system. When necessary, the sample extracts were diluted to the desired concentration. The mobile phase consisted of KH_2PO_4 (85 mM; pH 7.5) with acetonitrile (65:35).

Histological studies.—For the histological studies, the offspring (42 alevins at 775–838 degree-days) of the seven lake trout females for which the initial thiamine levels in the unfertilized eggs were measured by biochemical methods were randomly sampled. Progeny were divided into two groups, with high and low thiamine levels. The offspring ($n = 24$) of four females with mean total thiamine concentrations of 0.47, 0.66, 0.70, and 0.73 nmol/g wet egg mass (the S-LO-C group) and those ($n = 18$) of three females with mean total thiamine concentrations of 1.51, 1.61, and 3.03 nmol/g (the S-HI-C group) were examined. The alevins from the S-LO-C group had clear signs of EMS, disequilibrium, and/or spiral and erratic swimming. Live alevins, six for each female, were anesthetized with MS-222 (tricaine methanesulfonate; Argent Chemical Laboratories, Redmond, Washington) at a concentration of 75 mg/L. Three alevins were fixed immediately after anesthesia in Bouin's fluid for 72 h and three in 4% buffered formalin for 48 h. After fixation, fish were transferred to water (formalin fixation) and then to 75% ethanol until further processed.

Alevins at the swim-up stage were measured before fixation and after roughly 6 months, immediately prior to being embedded in paraffin. There was a significant correlation ($r = 0.976$; $P < 0.01$) between the body mass of "fresh" fish and that of fish after fixation for histological analyses at the swim-up stage. The body mass of fish used for histological examination was 24.4% smaller than that of fish evaluated directly (Table 1). Total length after fixation was 22.9 ± 1.8 mm and 24.3 ± 0.6 mm for the fish in the S-LO-C and S-HI-Cgroups, respectively.

All alevins were paraffin-embedded after dehydration with a graded ethanol series and treated with xylene. Histological sections were cut transversally into a complete series of serial sections (5 μm) at the head (brain and eye) and the thorax (liver) levels and mounted on albumin-coated slides. Sections were stained using three different methods. Every second slide with the head and thorax was stained with Mayer's hematoxylin and eosin (H&E) for topographic histological analysis. In addition, the (Alcian Blue/Periodic Acid Schiff; pH 2.5; counterstained with Gill's hematoxylin) procedure was carried out to identify glycogen (Merck & Co., Whitehouse Station, New Jersey) on every remaining transverse section in the area where the liver was present. In the case of the AB/PAS staining, the PAS-positive reaction of Goblet cells in the stomachs of alevins was used as the control for staining results. In all samples the presence of Goblet cells with PAS-positive reaction in the stomach epithelium was confirmed. Sections of the brain were stained with cresyl fast violet (Nissl staining) at pH 2.5, according to the procedure of William Beaumont Hospital (www.histosearch.com//histonet.html). Staining with cresyl fast violet was used to demonstrate the position of nuclei and the distribution of Nissl granules in the cell cytoplasm. Analysis of the structure of the body wall muscle fibers was conducted on the sections fixed in Bouin's fluid and stained with H&E.

To compare the glycogen content of the hepatocytes, a semiquantitative scoring system was used. A detailed description of the results of the histochemical staining (AB/PAS) was made on the basis of the following scoring system: 0 = no glycogen (no PAS-positive reaction); 1 = a small amount of glycogen (PAS-positive reaction pronounced in hepatocytes: an insignificant number of glycogen particles in the view field [63× objective], very weak magenta color detectable); 2 = an average amount of glycogen (stronger PAS-positive reaction: numerous glycogen particles in the view field, with a distinct magenta color); 3 = a large number of glycogen particles in hepatocytes (very strong PAS-positive reaction: intense magenta color, with numerous small and large glycogen particles in the cytoplasm). The overall degree of the glycogen density was calculated by averaging the scores of the results for the S-LO-C and S-HI-C groups. The histological sections were observed under light microscopy. Micrographs were taken with a microscope (Zeiss Axioscope, Zeiss, Germany) and a digital camera (Olympus MagnaFire Digital).

Feeding trial.—Alevins were kept in 40-L glass aquaria with aeration and a water flow of approximately 1 L/min. Fish were fed a commercial diet (AgloNorse, K/S Tromsø Fiskeindustri A/S, Tromsø, Norway) by hand five times per day to near satiation after the swim-up stage. At around 1,500 degree-days (range, 1,514–1,532), the density of the surviving lake trout juveniles was readjusted to 50 fish/tank. All of the six readjusted groups (H-LO-T, S-LO-T, H-HI-T, H-HI-C, S-HI-T, and S-HI-C) were fed a commercial diet at 3.1% of BM twice a day (0900 and 1600 hours) 7 d a week. Experimental tanks were cleaned by siphoning every morning to remove feces. All fish were weighed at the beginning and end of the feeding experiment (32 d) to calculate BM gain.

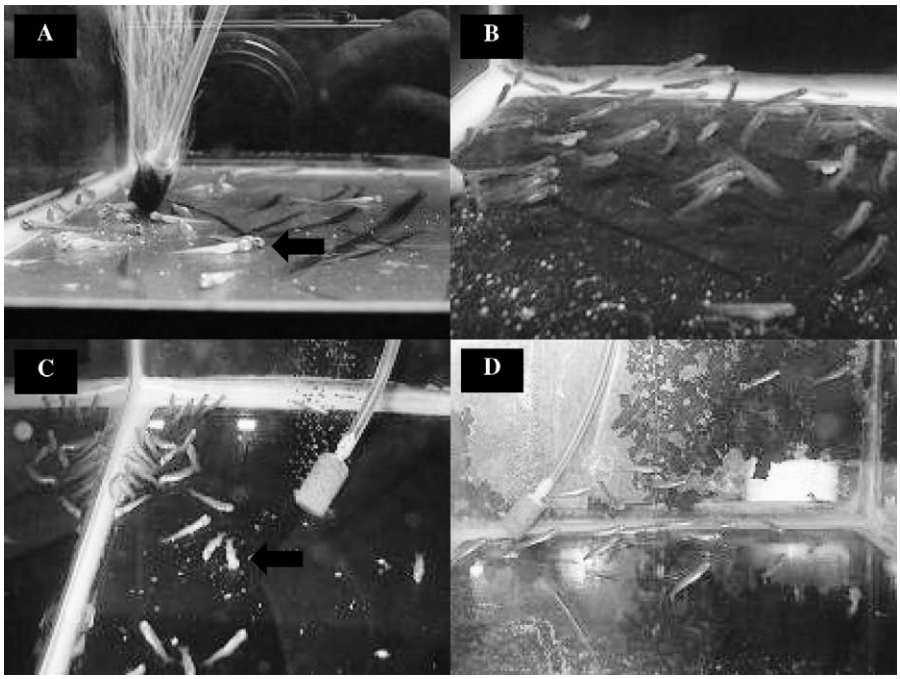

FIGURE 3.—Panels **(A)** and **(C)** show lake trout alevins from the control (untreated) low-thiamine groups; panels **(B)** and **(D)** show alevins from H-LO-T and S-LO-T group, respectively. The arrows in **(A)** (progenies of female 1; Table 1) and **(C)** (progenies of female 11; Table 1) indicate loss of equilibrium in alevins. The photographs were taken 2 d after the 50% swim-up stage.

Statistics.—The influence of the treatments was compared by means of one-way analysis of variance (ANOVA) in SPSS, version 12.0. Data expressed in percent were arcsine transformed before the significance of differences was calculated. Mean values of particular groups were compared via Duncan's multiple-range test. The results were regarded as significant for semiquantitative scoring system used in histochemical studies at $P < 0.01$. Data are presented as means ± SDs. Correlations and *P*-values of paired data were compared by *t*-tests.

Results

Initial Egg Viability and Thiamine Concentration

There was no evidence of correlation between mortality prior to the eyed embryo stage and the thiamine concentration in the unfertilized eggs ($R^2 = 0.027$; $P = 0.586$; Table 1). The origin of fish or size of females seemed to play no role in egg viability and did not affect thiamine concentration. The differences in total thiamine concentrations in unfertilized eggs and survival at the eyed embryo stage were not significantly different between the southern and northern sites ($P = 0.232$ and 0.187, respectively). When the progeny were divided into four groups (Table 1) according to thiamine concentration and time of treatment, there were significant differences among the groups ($P = 0.013$). However, survival was not different among the groups ($P = 0.185$).

Survival of Embryos and Alevins in Relation to Thiamine Concentration

Lake trout reached the eyed embryo stage at 290–310 degree-days and started to hatch at 516–569° degree-days. The swim-up stage was reached at 775–838 degree-days, and 16 d of feeding was associated with the attainment of 917–980 degree-days.

The thiamine hydrochloride concentration of the buffered thiamine solution was stable during the thiamine immersion experiments and amounted to 2.01 ± 0.06 to 2.13 ± 0.01 g/L between 0 and 270 min following preparation. Therefore, we can conclude that the alevins at both the 50% hatching and 50% swim-up stages were exposed to the desired concentration of thiamine. The thiamine treatment was effective in the LO group based on visual observations (Figure 3), and EMS symptoms were completely absent in the treated groups. The survival of alevins correlated with their thiamine concentration or the effects of thiamine treatment (Table 2). Proportional

TABLE 2.—Mean ± SD survival between hatching and swim-up and between swim-up and 16 d after swim-up of lake trout treated with a thiamine solution (T) or not (control [C]). See Table 1 for basic group designations. Different letters within a column indicate significant differences ($P < 0.05$).

Group	Survival	
	Swim-up[a]	16 d after swim-up[b]
H-LO-C	83.2 ± 11.3 y	0.0 ± 0.0 x
H-LO-T	97.7 ± 2.9 z	79.8 ± 13.7 y
H-HI-C	94.6 ± 6.9 z	99.3 ± 1.0 z
H-HI-T	97.0 ± 1.1 z	100.0 ± 0.0 z
S-LO-C	91.0 ± 7.1 zy	5.1 ± 10.3 x
S-LO-T	91.0 ± 7.1 zy	89.5 ± 11.9 zy
S-HI-C	96.2 ± 2.7 z	99.3 ± 1.2 z
S-HI-T	96.2 ± 2.7 z	98.8 ± 0.8 z

[a] 100 × number of progeny at swim-up/number of progeny at hatching stage.
[b] 100 × number of progeny 16 d after swim-up/number of progeny at swim-up.

survival between the hatching and swim-up stages was lower for the H-LO-C group than for the H-LO-T group or any of the high-thiamine groups but not significantly different from that for the S-LO-C or S-LO-T groups (Table 2). Thiamine treatment of both low groups (H and S) resulted in higher survival between swim-up and 16 d after swim-up than in the low control groups ($P < 0.05$), while there was no effect of treatment in the high-thiamine groups (Table 2). Untreated lake trout alevins having a low total thiamine level in eggs (0.46–0.73 nmol/g of tissue; Table 1) exhibited EMS resulting in 100% mortality during the period from swim-up to 16 d after swim-up (120.4 degree-days for the H-LO-C group and 106.7 degree-days for the S-LO-C group; Table 2). There was one exception in the S-LO-C treatment, one group of fish experiencing 20.5% survival at 16 d after swim-up.

The proportion of free thiamine in lake trout tissue has a tendency to decrease with the age of fish (Table 3). In other words, the proportion of phosphorylated thiamine in fish tissues increases as the fish age. Fish in the S-LO-T and S-HI-T groups showed higher levels of free thiamine than the untreated controls. Among the H groups there was no difference in total thiamine level between treated and control fish (Figure 4A), while among the S groups there was a significant difference related to the effect of thiamine treatment (Figure 4B) directly after treatment (1 h). The *P*-values for the comparisons between treated and control fish in the H-LO, H-HI, S-LO, and S-HI groups were 0.985, 0.796, 0.003, and 0.057, respectively. At the end of the feeding experiment, there were no significant differences in BM gain and survival ($P > 0.05$; Table 4). All treatments had survival rates above 91.6% in the feeding trial.

Histological Analysis of Swim-Up Alevins

The brains of all sampled alevins from the S-HI-C group showed well-defined structure with unchanged

TABLE 3.—Concentration (nmol/g of tissue) and percentage of free thiamine (T), thiamine monophosphate (T-mp), thiamine diphosphate (T-dp), and total thiamine (TT) in the unfertilized eggs, eyed embryos, and swim-up-stage alevins of lake trout. Different letters within a row indicate significant differences ($P < 0.05$).

Thiamine compound	H-LO		H-HI		S-LO		S-HI	
	Concentration	%	Concentration	%	Concentration	%	Concentration	%
Unfertilized eggs								
T	0.37 ± 0.09 y	63.1	2.89 ± 1.70 zy	78.5	0.37 ± 0.09 y	56.9	1.50 ± 0.92 z	69.7
T-mp	0.06 ± 0.03 y	11.1	0.25 ± 0.09 z	8.7	0.06 ± 0.02 y	9.5	0.20 ± 0.02 z	10.9
T-dp	0.15 ± 0.02 x	25.8	0.32 ± 0.07 zy	12.8	0.21 ± 0.04 yx	33.7	0.35 ± 0.14 z	19.4
TT	0.58 ± 0.11 y	100.0	3.46 ± 1.84 z	100.0	0.64 ± 0.12 y	100.0	2.05 ± 0.85 zy	100.0
Eyed embryos								
T	0.14 ± 0.08 y	50.1	2.40 ± 1.59 z	81.4	0.11 ± 0.01 y	37.9	0.90 ± 0.69 zy	73.0
T-mp	0.03 ± 0.01 y	11.2	0.14 ± 0.07 z	5.8	0.04 ± 0.01 y	14.5	0.09 ± 0.02 zy	9.2
T-dp	0.10 ± 0.02 y	38.7	0.25 ± 0.08 z	12.8	0.14 ± 0.02 y	47.6	0.18 ± 0.05 zy	17.9
TT	0.28 ± 0.07 y	100.0	2.79 ± 1.74 z	100.0	0.29 ± 0.04 y	100.0	1.17 ± 0.76 zy	100.0
Control alevins analyzed at swim-up								
T	0.03 ± 0.01 y	8.8	0.18 ± 0.02 z	13.9	0.03 ± 0.01 y	9.6	0.06 ± 0.04 y	9.4
T-mp	0.31 ± 0.12	91.2	0.38 ± 0.11	58.7	0.21 ± 0.04	90.4	0.22 ± 0.10	69.1
T-dp		0.0	0.35 ± 0.03	27.4		0.0	0.14 ± 0.15	21.5
TT	0.34 ± 0.13 y	100.0	0.78 ± 0.38 z	100.0	0.23 ± 0.03 y	100.0	0.35 ± 0.09 y	100.0
Thiamine-treated alevins analyzed at swim-up								
T	0.20	10.2	0.15 ± 0.08	16.8	1.09 ± 0.47	77.0	3.10 ± 2.17	74.4
T-mp	0.30 ± 0.13 y	85.0	0.45 ± 0.02 z	58.6	0.21 ± 0.07 y	16.6	0.21 ± 0.03 y	6.9
T-dp	0.03	4.9	0.22 ± 0.15	24.5	0.12 ± 0.02	6.4	0.43 ± 0.27	18.7
TT	0.37 ± 0.23 y	100.0	0.81 ± 0.23 y	100.0	1.39 ± 0.47 y	100.0	3.73 ± 1.93 z	100.0

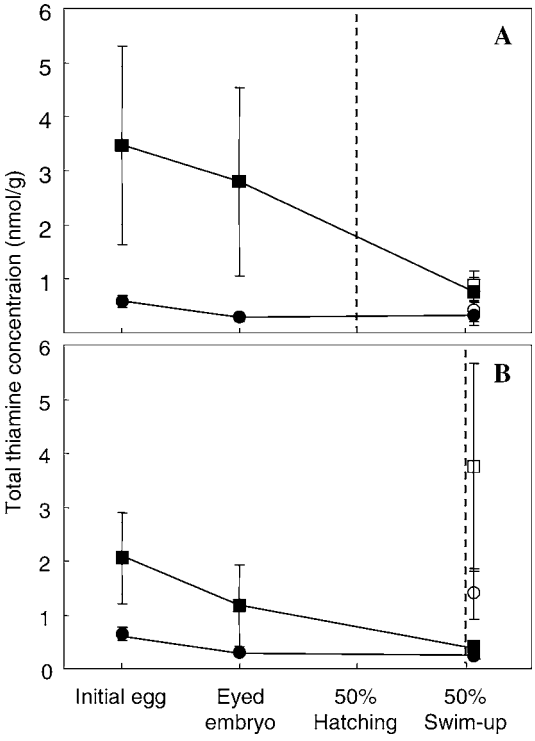

FIGURE 4.—Total thiamine concentrations of lake trout alevins treated with a thiamine solution (open symbols) and untreated controls (filled symbols) in HI (squares) and LO (circles) groups at **(A)** 50% hatching and **(B)** 50% swim-up stage. The dotted vertical lines indicate the times of thiamine treatment. The differences between untreated and treated fish were not significant for the H-LO ($P = 0.624$), H-HI ($P = 0.728$), and S-HI ($P = 0.098$) groups. The difference was significant between treated and untreated fish for the S-LO group ($P = 0.015$).

cells in each stratum (Figure 5A, B). To the contrary, in 2 alevins (out of 24) from the S-LO-C group, the cresyl fast violet staining revealed advanced pathological changes in the olfactory lobe (Figure 6A) and in all strata of the whole brain (Figures 6B; 7A, B). The lesions had the form of rounded, shrunken neurons with an abnormal distribution of the Nissl granules (dark staining) and large interstitial spaces, indicating edema. In particular, many necrotic cells were present in the brain mantle and the marginal layer. Pathological neurons were less frequently observed in the ependymal layer. In addition, in one alevin with advanced degeneration of the nervous tissue altered neurons were also evident in the retina; by contrast, there were no changes in the eyes of alevins from the S-HI-C group (Figure 8A, B). In the other alevin from the S-LO-C group with pathological changes, a few necrotic cells were noticed in a mantle of the mesencephalon (Figure 9). It seems that in this

TABLE 4.—Body mass gain and survival of lake trout juveniles (initial thermal age of 1,500 degree-days) fed a commercial diet for 32 d. There are no significant differences ($P > 0.05$) among treatments.

Treatment[a]	Body mass gain (%)	Survival (%)[b]
H-LO-T	64.0 ± 16.2	96.5 ± 4.1
H-HI-T	50.0 ± 34.2	91.6 ± 1.9
H-HI-C	56.5 ± 21.2	93.5 ± 4.4
S-LO-T	58.3 ± 15.1	99.0 ± 2.6
S-HI-T	82.8 ± 40.4	93.3 ± 5.0
S-HI-C	98.1 ± 34.5	94.7 ± 5.0

[a] The H-LO-C and S-LO-C treatments were excluded because of 100% mortality.
[b] 100 × number of progeny 32 d after 1,500 degree-days/number of progeny at 1,500 degree-days.

alevin the changes were in the initial stage, as they appear in an insignificant number of pathological cells and their occurrence is limited to certain areas.

The AB/PAS staining of the liver revealed the variable content of glycogen (1.6 ± 1.3 according to the scoring system) in the hepatocytes of the S-LO-C group. Glycogen was not present in the livers of five alevins from this group with egg thiamine levels of 0.70 nmol/g and in two alevins with egg thiamine levels of 0.73 nmol/g (Figure 10A). The lack of glycogen was associated with the pathological changes in the nervous system described earlier. For the remaining individuals from the S-LO-C group, the extent of glycogen deposition was variable (grade 1, 2, or 3). The glycogen content of the hepatocytes of all alevins from the S-HI-C group was high (3 ± 0.0; Figure 10B). The difference in the glycogen content between the two groups was statistically significant ($P < 0.01$). We examined the structure of the liver for heterogeneity in the size and shape of the nuclei or cells, the infiltration of hepatocytes with lipids, an unnoticeable border between cells, and the presence of inflammatory or necrotic cells. No pathological changes in liver structure were found in the studied groups (Figure 10A, B). No changes in glycogen density or the structure of the body muscles were detectable in any sampled alevins of the S-LO-C and S-HI-C groups.

Discussion

The thiamine concentrations found in the unfertilized eggs of Lake Michigan lake trout in 2006 (1.74 ± 1.59 nmol/g) are at the very low end of those found in lake trout from Lake Ontario over the period from 1994 to 2001 (Fitzsimons et al. 2007). If it is assumed that the threshold for 50% mortality due to EMS is a total thiamine concentration in the eggs of 1.57 nmol/g (Fitzsimons et al. 2007) (0.8 nmol/g when expressed as free thiamine; Brown et al. 1998), the Lake Michigan

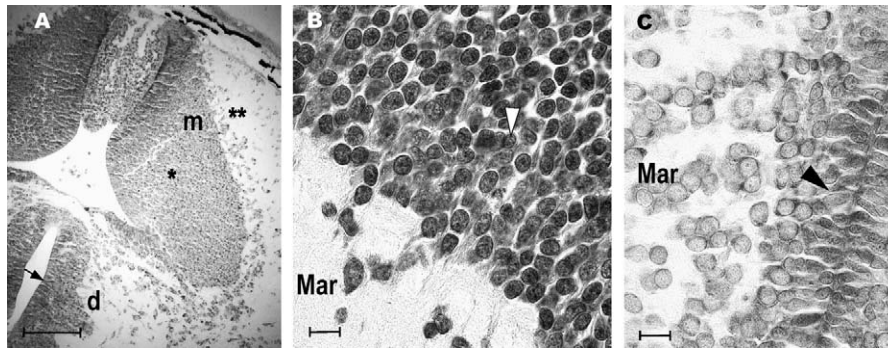

FIGURE 5.—Panel **(A)** shows a cross-section of the mesencephalon (m) and diencephalon (d) of an alevin from the S-HI-C group. No pathological changes are evident in the ependymal, mantle (*), or marginal (**) layers. Panel **(B)** provides a higher magnification of the mesencephalon showing all unchanged neurons in the mantle layer (white arrowhead). Panel **(C)** provides a higher magnification of the telencephalon showing all unchanged neurons in the marginal (Mar) and ependymal (black arrowhead) layers. The scale bars represent 100 μm in (A) and 50 μm in (B) and (C); cresyl fast violet stain was used.

lake trout population we examined is at extremely high risk of the disease.

The LO groups showed common EMS symptoms, such as dark coloration, uncoordinated, erratic patterns of swimming, and loss of equilibrium. This was followed by high mortality within 1–2 weeks. The increased survival of EMS-afflicted lake trout after thiamine immersion was significant independently of the time of treatment (50% hatching or 50% swim-up; Table 2). However, the major finding of this experiment was that thiamine immersion can be delayed until the swim-up stage, when recovery, measured as survival during the active feeding phase until day 16, was 79–100%. This was not the case for fish treated at the 50% hatching stage. Our results corroborate the finding of Barnes et al. (2001), who reported initial observations of thiamine hydrochloride treatment of eggs of landlocked fall Chinook salmon *Oncorhynchus tshawytscha* treated immediately after fertilization (i.e., at the egg hardening stage). Thiamine treatments at

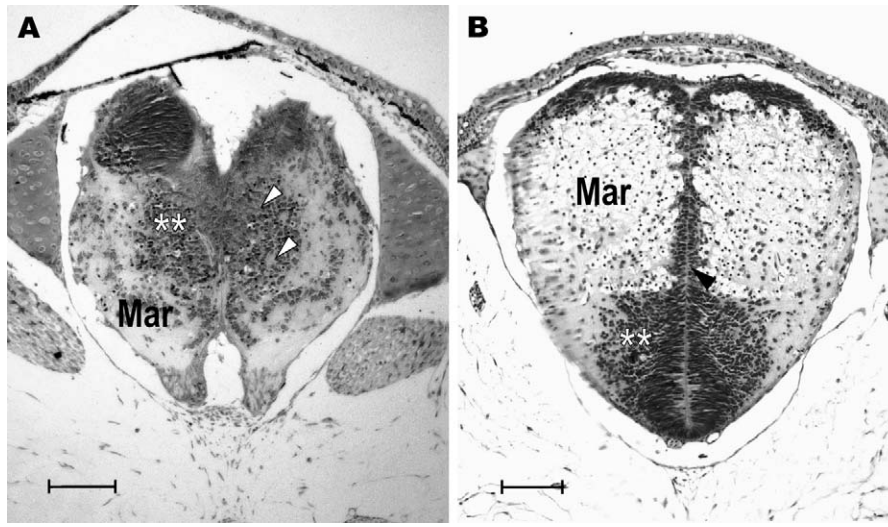

FIGURE 6.—Panel **(A)** shows a cross-section of the olfactory lobe of an alevin from the S-LO-C group. Necrotic cells (black round spots marked by white arrowheads) are easily distinguishable in the mantle (**) and marginal (Mar) layers. Panel **(B)** shows a cross-section of the anterior telencephalon of an alevin from the same group with advanced pathological changes in the marginal layer. Necrotic, shrunken cells are visible in the whole stratum, with large interstitial spaces between them. Necrotic neurons in the mantle layer are visible as small black points. The black arrowhead shows the ependymal layer. The scale bar represents 100 μm in each panel; cresyl fast violet stain was used.

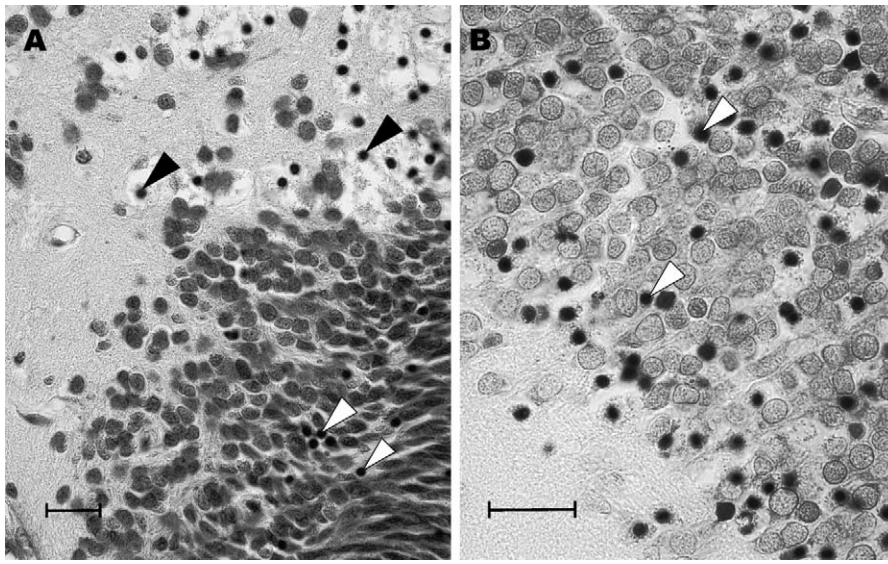

FIGURE 7.—Panel **(A)** provides a higher magnification of the anterior telencephalon shown in Figure 5B; and **(B)** shows the mesencephalon of the alevin from the S-LO-C group with advanced pathological changes. Necrotic neurons are visible in the marginal layer (black arrowheads) and in the mantle layer (white arrowheads). To compare these with normal structure of the telencephalon and mesencephalon (S-HI-C group), see Figure 5B, C. The scale bars represent 20 μm in each panel; cresyl fast violet stain was used.

concentrations of 250 and 1,000 mg/L resulted in insignificant increases (1–2%) in embryo survival from the eyed embryo stage to hatching. No differences in percent survival were observed in controls until 28 d after the swim-up stage. In the current study, thiamine treatment of yolk sac alevins at a concentration of 2,000 mg/L of thiamine hydrochloride resulted in increases in proportional survival between swim-up

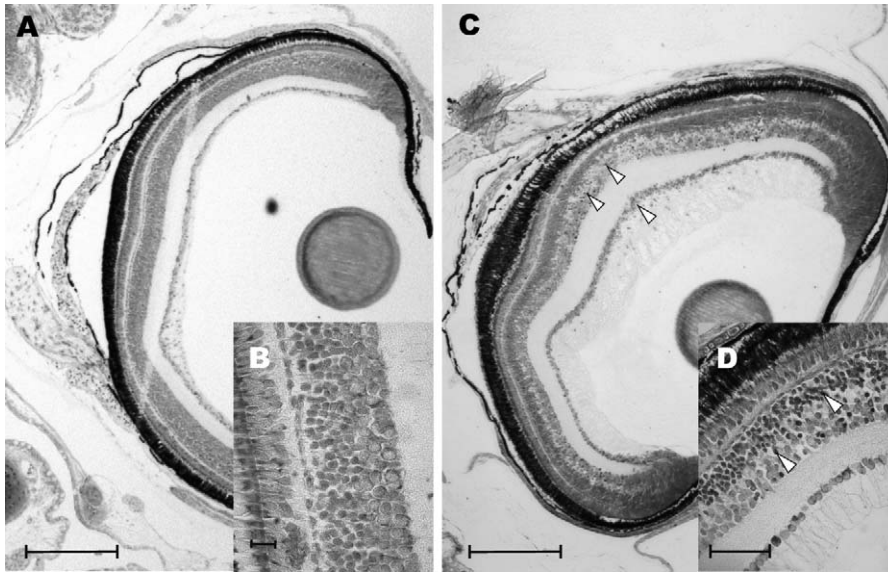

FIGURE 8.—Panels **(A)** and **(B)** show longitudinal sections of the eye of an alevin from the S-HI-C group, panels **(C)** and **(D)** similar sections from an alevin from the S-LO-C group. The arrowheads in the last two panels indicate necrotic cells in the retina. The scale bars represent 500 μm in (A) and (C), 20 μm in (B), and 50 μm in (D); cresyl fast violet stain was used.

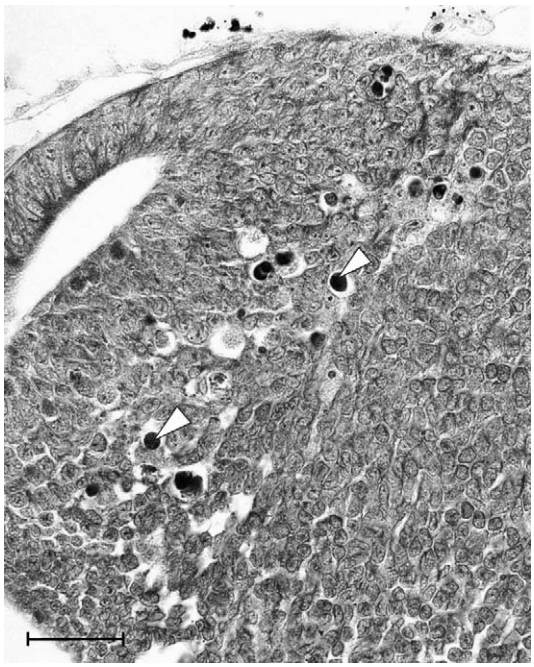

FIGURE 9.—Cross-section of the mantle layer in the mesencephalon of an alevin from the S-LO-C group, with initial changes (necrotic cells [arrowheads]) in the nervous system. To compare this with normal structure of the mesencephalon, see Figure 5b. The scale bar represents 30 μm; cresyl fast violet stain was used.

and 16 d after swim-up of 43–84%. Treatment at this concentration for 2 h was enough to eliminate the occurrence of EMS. Similarly, Fisher et al. (1998) indicated that Atlantic salmon yolk sac fry subjected to thiamine baths between 5 and 11 weeks after hatching almost completely avoided avitaminosis-related mortality.

The free-thiamine levels of alevins treated with thiamine at the swim-up stage were dramatically higher than those of untreated groups (Table 3; Figure 4). In alevins treated at the 50% hatching stage, no differences were noticed between the treatment and control groups several days later (Figure 4). Amcoff et al. (2002) concluded that immersion treatment of Atlantic salmon at the yolk sac absorption stage resulted in an increase of total thiamine in fish of 0.31–1.7 nmol/g. However, the authors argued that this increase was apparently not sufficient to mitigate the deficiency of the vitamin, and high mortality (82%) followed. Our results somewhat contradict previously reported data, although differences in fish species and time of treatment make direct comparisons impossible. In the present study, the total thiamine level increased 10-fold, and the major portion of accumulated thiamine in the fish body was of the free form (Table 3). It is well established that in mammalian systems the penetration of thiamine into the central nervous system via the blood–brain barrier is carrier mediated and nonlinear, although extremely fast (Lockman et al. 2003). Therefore, we interpret the current results as follows. As the metabolic rate of swim-up-stage alevins increases over 10-fold relative to that of newly hatched alevins (Terner 1968), the increase in oxygen consumption will correspond to the enhanced uptake of thiamine and subsequently have a major and rapid impact in reversing thiamine deficiency. Bettendorff et al. (1997) demonstrated that it takes only 1 h for human neuroblastoma cell mitochondria to recover their normal structure and for the thiamine-diphosphate-dependent enzyme activities that induce normal oxygen consumption to be restored. Therefore, in vitro studies and our observations in vivo are complementary and appear to support the conclusion of the high efficiency of thiamine treatment at the 50% swim-up stage.

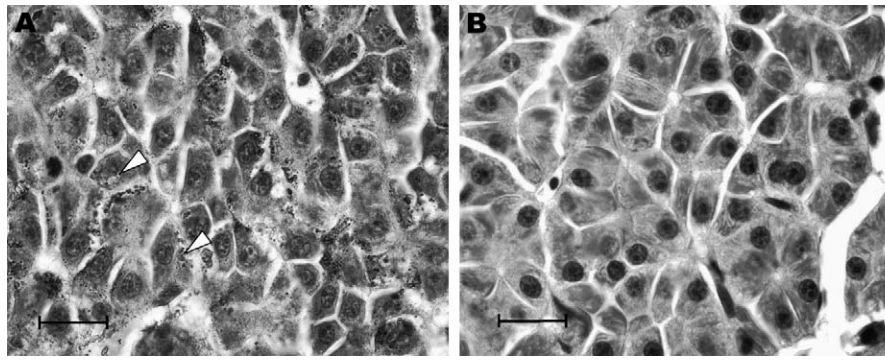

FIGURE 10.—Panel **(A)** show an image of the liver tissue found in all healthy alevins from the S-HI-C group and 17 of the 24 from the S-LO-C group; glycogen droplets (white arrowheads) are evident. Panel **(B)** shows the absence of glycogen granules from the hepatocytes of the other 7 alevins from the S-LO-C group. The scale bars represent 20 μm; AB/PAS stain was used.

In light of the observations by Fisher et al. (1998) that mass mortality followed gross pathological changes and erratic behavior within 1–2 weeks in Atlantic salmon alevins, it seems logical that until such a threshold is reached, the frequency of pathological changes in lake trout is not overwhelming and their intensity increases gradually. Pathologies first appear in the liver (lack of glycogen) and only secondarily in highly innervated tissues such as the olfactory lobe, central nervous system, and retina (Figures 5–9).

The free thiamine in lake trout tissues showed a tendency to be converted to phosphorylated forms during early developmental stages (i.e., from unfertilized egg to eyed embryo to alevin). Dakshinamurti and Chauhan (1994) have suggested that egg yolk thiamine binding protein plays a role in the transport of free thiamine to the developing embryo. Likewise, the majority of free thiamine in lake trout eggs at the beginning of development might be bound with protein.

The present study showed that low thiamine levels in unfertilized eggs led to pathological changes in the brain, olfactory lobe, and liver of two lake trout alevins at the swim-up stage as well as—for the first time—in the retina. No glycogen deposits were found in seven alevins from the S-LO-C group. Increased necrotic and hydropic cell degeneration in the brain and reduced hepatic and muscle glycogen levels were also observed in Atlantic salmon yolk sac fry suffering from M74 syndrome that developed from natural causes or was stimulated by thiamine antagonists (Amcoff et al. 1999, 2002; Lundström et al. 1999). In the present study, lake trout alevins showed no alteration in hepatocyte structure as described by Amcoff et al. (2002), that is, no "condensed marginated chromatin, a translucent nucleosol, and condensation of the nucleolus" were seen. These data confirm that the alterations in lake trout alevin brain and liver were connected with thiamine deficiency. It can be supposed that the health status of lake trout alevins with low thiamine egg levels but no histopathological changes corresponds to that of Atlantic salmon fry in the preclinical stage of M74, with no lesions in their tissues (Lundström et al. 1999). The exception would be alevins with lesions in the brain and lower glycogen deposits in the hepatocytes, which would correspond to the Atlantic salmon yolk sac fry at the clinical stage described by Lundström et al. (1999). According to these authors' classification, there were no lake trout alevins with a terminal or late stage of disease characterized by the simultaneous presence of severe alterations in the brain, hepatocellular necrosis, and hyaline degeneration of the skeletal muscles. However, the comparison with Lundström et al. (1999) is not precise because those authors did not provide data on thiamine levels in the sampled fry.

Similar changes in the nervous system (necrotic cells in the brain layers and disturbed development of the retina) of lake trout alevins affected by thiamine deficiency were observed in embryos of rainbow trout embryos *Oncorhynchus mykiss* exposed to oxytetracycline (Arias et al. 2002). On the contrary, no changes in the brains of M74-affected Atlantic salmon alevins were observed, whereas lower glycogen levels and increased vacuolization of hepatocytes were noticed in suffering fish (Norrgren et al. 1993). It is worth emphasizing that other environmental contaminants (e.g., pesticides) may act in concert with thiamine deficiency (Skea et al. 1985) and result in mortality that is difficult to separate from EMS or M74 disease. Thiamine deficiency influences the cellular carbohydrate metabolism in the brain and liver and causes neurological disorders (Huang et al. 2007) similar to those described in detail in mammals (Mulholland 2006). It can be concluded that liver alterations appear before symptoms in the brain. That means that fast liver recovery and an increase in glycogen storage after thiamine treatment is very possible. Metabolic disorders at the mitochondrial level in neuroblastoma cell culture have been shown to be completely reversible (in terms of the respiration rate) within 1 h of thiamine treatments (Bettendorff et al. 1995). It is likely that thiamine treatment at the swim-up stage, when the metabolic rate is much higher than in newly hatched alevins, will prevent subsequent changes in the central nervous system.

In future work, we would like (1) to compare the histopathology of EMS-affected swim-up alevins before and after thiamine treatment. Furthermore, we need (2) to confirm that pathological changes are not occurring at the hatching stage and that the first signs of nervous system pathologies are recognizable at a later stage.

Acknowledgments

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