GREAT LAKES FISHERY COMMISSION

2004 Project Completion Report[1]

Understanding Variabilities in Lamprey Gametes Quality in Relation to Availability of Nutrients in Host Fish

by:

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1. Introduction

The effect of availability of nutrients in diets of salmonid fish in the Great Lakes on their offspring viability was well documented. In particular, the effect of vitamins, thiamin and tocopherol had a major impact on embryonic and early life stages survival. However, there is no data on possible impacts on gamete biochemical characteristics in lamprey and viability of their eggs or fertilizing ability of the sperm. The invasion of sea lamprey into freshwater lakes resulted in significant changes in dietary sources of nutrients during parasitic stages of lamprey. Marine fish contain significantly higher levels of thiamin (vitamin B_1) and tocopherol (vitamin E) in tissues and blood than their freshwater counterparts. Consequently, sea lamprey in the Great Lakes are likely to suffer from deficiencies of some essential nutrients. Taking into account that lamprey are on the top of the ecological pyramid, the nutrients in short supply will most severely affect the parasitic form. Therefore, the objectives of this proposal were to evaluate concentrations of thiamin and tocopherol in ova and sperm of lamprey from Great Lakes (freshwater origin) and Cocheco River, Dover, New Hampshire (marine origin) and correlate with gametes viability.

Understanding the nutrient-regulated mechanism of success in reproduction of the sea lamprey is a prerequisite for further attempts to predict population dynamics. If we are able to show association between concentration of specific nutrients and total number of viable eggs produced by the stock in a particular year, these data can be used to predict recruitment of lamprey. In other words, accurate estimation of environmental effects on the total biomass of mature parasitic forms is essential to develop a rational basis for lamprey stock reduction.

2. Background

2.1. Early mortality syndrome (EMS) in salmonids

Significant changes in the reproductive capacity of salmonid populations in Lake Michigan and Lake Ontario and, to lesser extent, Lake Huron and Erie have occurred in the 1990's due to dramatically increased mortality in the hatcheries. The Great Lakes Fishery Commission sponsored two workshops to facilitate more extensive investigations into the cause(s) of this early mortality syndrome (EMS). This mortality was observed in feral broodstock salmonids under culture situations, and most likely contributes to the limited natural reproduction of lake trout. EMS was also identified as the cause of decreased fry survival of salmonids from Cayuga Lake, New York (Fisher et al. 1995). Fisher et al. (1996) concluded that the catastrophic mortality in salmonids from the Finger Lakes and lake trout from Great Lakes was directly linked to reduced thiamin concentrations in eggs. Low concentrations of thiamin in the adult diet (composed mostly of alewife) are responsible for vitamin B_1 deficiency. M-74 syndrome reported in two salmonid species from the Baltic Sea region in Europe, brown trout and Atlantic salmon, seems to be very similar to EMS. Furthermore, Bylund and Lerche (1995) found that eggs or fry treated with thiamin solution significantly improved their survival.

2.2. Thiamin deficiency

Thiamin deficiency was recorded in fish fed in captivity on diets composed of anchovy (Ishihara et al. 1978). Specifically designed semi-purified diets deficient in thiamin (Morito et al. 1986) resulted in deficiency signs which included irritability, spiral swimming and anorexia. Neurological signs of thiamin deficiency occurred in salmonids at 4-5 weeks, indicating that younger (smaller) fish are much more sensitive to a thiamin deficiency than older (larger) fish. Furthermore, Amcoff et al. (1998) observed in Atlantic salmon abnormal behaviour of females

such as wiggling, sideways swimming and lack of coordination. These symptoms were linked to disturbances in swim bladder inflation and deaths.

2.3. Lipid-soluble vitamin, tocopherol

An essential role of highly unsaturated fatty acids (HUFA) in ovarian development and viability of embryos was reported in several species of marine fish (Verakunpiriya et al. 1996). Simultaneously, presence of antioxidants in the diet is considered essential to maintain integrity of structural phospholipids in salmonids fed diets high in HUFA (Cowey et al. 1983). The enhanced requirement for tocopherol during embryonic development and utilization of the yolk sac in salmon (Cowey et al. 1985) was demonstrated. Although, the parallel evidence of high correlation between vitamin E status and lipid oxidation in both maternal and in the offspring tissues resulting in severe malformation in newly born mice (Siman 1997) was not demonstrated in fish, the effect of low dietary tocopherol on high lipid oxidation in the condition of hyperoxic stress was shown in salmon (Lygren et al. 2000). Tocopherol is specifically transported from muscle and liver to ovaries during gametogenesis in fish (Hamre et al. 1994) and in fish characterized by partial spawning tocopherol concentration in eggs decreases during spawning season (Verakunpiriya et al. 1996). The evidence of vitamin E concentrations in livers of adult lake trout from L. Ontario suggest that females whose offspring had EMS had also less than half of vitamin E content compared to the group without EMS. However, there was no significant difference in vitamin E in embryos of trout with or without EMS (Palace et al. 1998).

Tocopherol supplement provided human sperm with dose-dependent protection against oxidant induced DNA damage (Donnelly et al. 1999) which can be interpreted as decreasing genotoxicity. Our studies on yellow perch (*Perca flavescens*) fed a vitamin E-deficient diet demonstrated that the level of tocopherol in sperm plasma was significantly decreased and

viability of sperm compromised (Lee and Dabrowski, 2004).

2.4. Hypothesis

The problem of egg quality in lamprey in relation to essential nutrients has not been addressed. No information, to our knowledge, is available on thiamin status in feral populations, whereas corresponding studies were carried out in respect to vitamin C status during gonad maturation in sea lamprey (Moreau and Dabrowski 1998). Therefore, we hypothesize that the possible ethiology of the diseases affecting salmonids in the Great Lakes known as early mortality syndrome (EMS) may have even greater impact on the sea lamprey, *Petromyzon marinus*, a common parasite of salmonids. We are going to use sea lamprey, an important Great Lakes species as a model for elucidating possible reproductive disturbances resulting in low egg quality and high offspring mortality. We propose to carry out a series of samplings of sea lamprey in L. Erie, L. Ontario and L. Huron populations, which were characterized by graded levels of thiamin concentration in lake trout (*Salvelinus namaycush*) eggs (Fitzsimons and Brown 1998). We then will be able to determine if deficiency of thiamin (vitamin B_1) leads to reproductive disturbances and pathologies in the early life history of lamprey. Second, we assume that lipid-soluble vitamin, tocopherol may be also in short supply in parasitic lamprey diet and exacerbates mortality syndrome in embryonic stages of lamprey. These results on inter-population differences in the status of vitamin concentrations in lamprey gametes will also provide the basis for further environmental and contaminant related studies with lamprey populations and suggest possible scenarios that may result from changes in food chains.

2. Objectives

The main goal of this proposal was to evaluate a magnitude of changes among individual

lamprey and inter-populations differences in the concentration of thiamin and tocopherol.

Specifically:

2.1. To analyze concentration of thiamin and tocopherol in gametes of sea lamprey following natural spermiation or ovulation.

2.2. To evaluate fertilizing ability of sperm and ova from individual females and correlate with concentration of vitamins,

2.3. To compare inter-population differences in fertilizing ability and vitamins. Three populations, i.e. from Great Lakes (freshwater origin) and Dover River, New Hampshire (Atlantic, marine origin) were studied.

3. Materials and Methods

Females and males, 5-20 fish of each gender, from three different locations (L. Erie, L. Huron and Atlantic) were air shipped to Columbus or collected during a field trip (L. Erie). It was assumed that fish will be caught during the final stage of their spawning migration and consequently in a very similar physiological condition. Upon arrival, fish were sampled for tissues and the remaining maintained until spermiation or ovulation occured. Fish were kept in the system described earlier and gametes obtained and fertilization carried out (Ciereszko et al. 2000).

Reproductive endpoints analyzed in the first year included: time of spawn, condition factor (somatic weight/total length), gonado- and hepato-somatic indices, egg viability and time to hatch, embryo (2-cell stage, 5 hours) and larvae (hatching, 16-21 days) survival and morphological abnormalities. Thiamin and tocopherol concentrations in blood plasma and eggs were correlated. Thiamin concentrations in tissues and fertilizing ability of eggs were correlated.

3.1. Vitamin E analysis

All-rac-α-tocopherol (αT), RRR-β-tocopherol (βT), RRR-δ-tocopherol (δT), all-rac-α-tocopheryl phosphate diNa (αTP) and all-rac-α-tocoheryl acetate (αTA) will be purchased from Sigma Chemical Company (St. Louis, MO). The HPLC system comprised a Beckman 101B pump (Beckman Instruments, Inc., Palo Alto, CA), a 15-cm x 4.6-mm Shodex ODSpak F-411 column (Showa Denko K. K., Japan), a column heater and temperature controller (Bioanalytical Systems, Inc., West Lafayette, IN) set at 40°C, and a programmable Jasco FP-920 fluorescence detector (Jasco Corporation, Tokyo, Japan). The mobile phase consisting of 93% methanol, 6.5% water and 0.5% H_3PO_4 will be delivered at a flow rate of 1.4 mL/min. Tocopherols in fish tissues will be extracted with methanol containing H_3PO_4 and pyrogallol. Although the efficiency of acetone extraction was superior to that of methanol-H_3PO_4, acetone was not employed because it caused a large front peak and an erratic base line that interfere with the detection of tocopherols. Using the present method, five tocopherols; αTP, αT, βT, δT and αTA, were completely separated and detected simultaneously (Moreau and Dabrowski, unpublished). At least 94% of each tocopherol was recovered from fish tissues. Therefore, frozen tissues (~200 mg) will be accurately weighed and homogenized for 1 min (3 times, kept on ice between homogenization) in 4.5 mL methanol containing 1% H_3PO_4 and 0.45 mL 5% pyrogallol in methanol. The homogenate will be centrifuged at 2,000 g for 5 min at 4°C. The supernatant will be transferred to a 10-mL flask and the pellet homogenized again for 1 min (2 times) and spun as above. The supernatants will be combined and the volume adjusted to 10 mL with methanol. Ninety % of known amounts of αT (1.4 µg/mL) added to tissue samples were recovered in this procedure. Based on stability data we obtained at different temperatures (data not shown) the extracts can be stored at -20°C and assayed within 10 days. Prior to an injection the extract will be filtered through a 0.45-µm nylon encased syringe filter.

3.2. Thiamin analysis

High performance liquid chromatography (HPLC) analysis for free thiamin and its phosphate forms (mono- and di-phosphate) was slightly modified based on Mancinelli et al. (2003) and Brown et al. (1998). The major modification was made on HPLC column selection. For sample extraction, sea lamprey egg samples (approximately 200 μg) were added by 600 μl 2% TCA extraction solution, and gently homogenized for 10-15 seconds. The homogenized samples were cooked in boiling water bath for 5 min and cooled on ice for 10 min. After cooling, the samples were added by 600 μl of ice-cold 10% TCA solution, and vortexed briefly to mix well. Then, the samples were centrifuged at 14,000 x g for 15 min at 4 ^{o}C. The clear supernatants (1 ml) were transferred into glass test tubes (10 ml capacity). The sample extraction at this stage is stable for 72 h at 4^{o}C under darkness. To remove TCA and lipids, the sample extracts in the test tubes were washed with 4 volumes of ethyl acetate-hexane solution (3:2, v/v). The washed sample of 0.5 ml volume was then transferred into Eppendorf tube and oxidized to thiochrome by adding 25 μl 30 mM $K_3Fe(CN)_6$. To increase pH of the sample extracts, 25 μl 0.8 M NaOH was added. Then, the oxidized sample extracts were vortexed and filtered before injection into HPLC system. When it is necessary, the sample extracts were diluted for desired concentration. For blood plasma extraction, the homogenization step was omitted.

The HPLC system consisted of a delivery system pump (Model 506A, Beckman Instruments Inc., San Ramon, CA, USA) 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, Japan) Shodex column coupled with NH_2 packed guard column. Fluorescent detector (BAS, LC22C)

was set at 375 nm for excitation and at 430 nm for emission. Mobile phase was composed of potassium phosphate buffer (pH 7.5, 85 mM) + acetonitrile, (65:35, v:v). Flow rate was 0.5 ml per min.

Each external standard curve of free thiamin (TH), thiamin-monophosphate (TMP), and thiamin-pyrophosphate (TPP) was prepared using 1 mM each standard stock solution in 0.01 M HCl. Each standard concentration ranged from 1.0 to 100 nmol/l for linearity. Extraction recovery rates were 94.7 ± 3.0 % (n = 4) for TH, and over 100% for both TMP and TPP. For the recovery, known amounts of each TH, TMP, and TPP standard were added into running samples at the beginning of the extraction and followed by extraction procedure as described above.

4. Results

TH concentrations in sea lamprey egg samples ranged from 0.1 – 0.4 nmol/g. TMP and TPP concentrations were 0.15 – 0.6 nmol/g and 0.6 – 1.1 nmol/g, respectively (n=18). We found that the dominant form of thiamin was thiamin-diphosphate in sea lamprey eggs and constituted approximately 70% of total thiamin. Contrary to the thiamin forms in egg samples, the dominant form of thiamin in blood plasma was TH (free thiamin). The percentage of the TH (dominant form) to total thiamin was over 86% and TH concentrations ranged from 0.1 to 0.6 nmol/ml (n=16). In seminal plasma, similar trend was found as in blood plasma. TH concentrations in seminal plasma were between 0.2 and 0.4 nmol/ml (98.5 ± 1%) (n=4).

Table 1 summarizes results for females from all three locations sampled prior to ovulation. The only exception to a general pattern are high concentrations of total thiamin in blood and significantly different profile of thiamin moieties (free and phosphorylated forms). It would be premature to associate this difference exclusively with marine origin of lamprey.

Figure 1 illustrates the relationship between blood plasma and gamete thiamin concentrations. This association is generally reflective of interorgan transport of vitamins as lamprey ceased feeding prior to spawning migration. In lamprey of freshwater origin, the predominant form in blood plasma, unphosphorylated thiamin, may indicate that the process of ovarian deposition was already completed. Therefore, the level found in blood was low.

Over the course of two months (May-June), 15 females from over 80 were selected at the time directly preceding or at the time of ovulation and stripped of their eggs. In these circumstances, eggs had acceptable qualities (over 25% viability at 2-cell stage embryo) (Fig. 2). We observed a trend that may indicate a threshold of the blood plasma thiamin concentration which is characteristic for a high frequency of decreased eggs viability (Fig. 3B). Presence of active, mono- and di-phosphates of thiamin in blood plasma of lamprey of Atlantic origin suggests that fish were in an earlier stage of gametogenesis and vitamin is continuously absorbed (Table 1).

Tocopherol levels, to the contrary, were significantly lower in eggs of Atlantic lamprey (1.3 ± 0.6 ug/g) in comparison to lampreys from L. Huron (7.04 ± 0.27 ug/g) (Table 2). A similar trend was found in blood plasma (Table 3). A correlation was found between tocopherol levels in eggs and blood plasma of lamprey females (Fig. 4).

5. Discussion

Recent data collected in the field (O'Connor, 2000) and our observation in the laboratory suggest that significant mortalities of female lampreys occurs prior to spawning. This may indicate disturbances in gonad maturation and/or ovulation processes. Furthermore, the viability of lamprey ova in respect to their fertilizing ability following induced ovulation has shown

significant variation among individuals (Ciereszko et al. 2000). Sperm quality, i.e. fertilizing ability was less variable among sea lamprey from Great Lakes (Ciereszko et al. 2001).

By invasion into freshwater, sea lamprey exposed itself to great changes in the diet, particularly lipids and lipid soluble vitamins in comparison to the characteristics of "marine lipids". Contrary to our "educated assumptions", the level of tocopherol, vitamin E, the vitamin intimately involved in reproductive processes, was not significantly decreased in the lamprey's feeding on "freshwater diet", i.e. salmonid fish hosts. Concentrations of tocopherol in sperm of lamprey corresponded to a high level of this vitamin in freshwater teleost sperm (Lee and Dabrowski 2004).

It was documented that thiamin level in salmonids forced to feed on alewives and smelt was dramatically decreased in comparison to populations feeding on lake herring and yellow perch. We speculated that the syndrome of thiamin avitaminosis in salmonids will be magnified in lamprey feeding on their body fluids and tissues. This assumption was only marginally correct (Fig. 5), although the effect on viability of lamprey eggs, possible interannual changes documented in salmonids (Fisher et al. 1998) and walleye (Vandergoot et al. 2001) require further studies. Understanding of nutrient-regulated success in reproductive output in sea lamprey is a prerequisite for further work on accurate prediction of population dynamics. If we are able to show a strong association between concentration of thiamin and total number of viable eggs produced by the population, in a particular year, these data can be used to estimate recruitment of lamprey.

The data collected during this investigation, concentration of thiamin and tocopherol in sea lamprey tissues, are the first of this kind and of general interest to fisheries biologists and managers in the Great Lakes basin. These data may already provide predictive power in regard

to the relationship between biochemical characteristics of lamprey gametes and their viability. The threshold established, below 0.2 nmol/g of eggs (Fig. 2) can be referred to changes in food chains, prey fish composition of salmonids, or alternative host fish of sea lamprey. It indicates that alteration in biochemical make up of lamprey gametes and changes in their viability can be used in a predictive fashion.

In addition, the data on sea lamprey combined with information on salmonids and vitamin levels in food chains of the Great Lakes (Fitzsimons et al. 1998) are valuable contribution to understanding of vitamin dynamics at the organismal level and in modelling transfers at various trophic levels and overall functioning of the ecosystem. The phosphorylated thiamin in lamprey eggs prior to fertilization is a remarkable departure from the pattern observed in landlocked Atlantic salmon eggs (Fisher et al. 1998) or freshwater medaka (*Oryzias latipes*) (Hishida and Nakano, 1954). Salmonids, where the predominant form of vitamin B_1 is free thiamin (86%), differ significantly from the profile observed in lamprey and walleye (84-88%) (Vandergood et al. 2001). It was speculated that an active form of thiamin will be required in fish exhibiting fast rate of embryonic development, at relatively higher water temperatures than in salmonids.

6. References

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Table 1: Thiamin concentrations in eggs and blood plasma of sea lamprey prior to ovulation.

Origin	Total in eggs	Free T	Mono P-T	DI P-T	Total in blood plasma	Free T	Mono P-T	DI P-T
	(nmol/g)	Expressed in %			(nmol/g)	Expressed in %		
L. Huron (n=3)	1.60 ± 0.25	8.7 ± 8.3	23.6 ± 9.5	62.7 ± 14.3	0.33 ± 0.22	96.8 ± 2.7	2.3 ± 1.5	0.9 ± 1.2
L. Erie (n=4)	1.75 ± 0.48	4.5 ± 2.0	17.0 ± 2.1	78.4 ± 3.5	0.10 ± 0.02	86.6 ± 3.3	8.6 ± 3.6	4.8 ± 1.3
R. Dover Atlantic (n=3)	1.18 ± 0.30	8.3 ± 3.5	16.7 ± 3.0	74.9 ± 5.0	1.48 ± 0.74	18.0 ± 3.5	23.5 ± 13.0	58.8 ± 17.1

Table 2: Vitamins E concentrations in lamprey tissues (µg/g)

Tissue	Origin	Fish	αT	βT+γT	δT
Eggs prior to ovulation	Dover River	1	0.77	0.004	0.038
		2	1.94	0.003	0.077
		3	1.07	0.007	0.044
Eggs prior to ovulation	Lake Huron	1	7.29	0.008	0.094
		2	6.65	0.007	0.082
		3	7.08	0.012	0.085
		4	7.14	0.002	0.071
Ovulated		5	5.16	0.005	0.075
		6	5.99	0.006	0.092
		7	4.59	0.003	0.054
		8	5.08	0.006	0.074
		9	5.36	0.007	0.092
		10	6.46	0.009	0.115
		11	8.51	0.009	0.105
		12	6.99	0.005	0.101
		13	5.93	0.009	0.099
		14	6.84	0.011	0.132
		15	4.79	0.010	0.068
		16	6.36	0.011	0.089
Eggs prior to ovulation	Lake Erie	1	5.39	0.001	0.092
		2	6.69	0.006	0.107
		3	5.66	0.002	0.101
		4	4.74	0.002	0.072
Seminal plasma	Lake Huron	1	11.32	0.006	0.160
		2	8.54	0.018	0.018

Table 3: Concentration of vitamins E in blood plasma of lamprey (μg/mL)

Origin	Fish	αT	βT+γT	δT
Dover River	4	0.16	0.003	0.006
prior to ovulation	5	0.28	0.002	0.010
	6	0.16	0.007	0.006
Lake Huron	1	0.78	0.002	0.007
prior to ovulation	2	0.98	0.003	0.013
	3	0.81	0.002	0.006
	4	0.73	0.001	0.006
ovulated	5	0.98	0.002	0.013
	6	0.27	0.001	0.003
	7	0.87	0.001	0.003
	8	0.72	0.001	0.007
	9	0.18	0.001	0.002
	10	1.04	0.003	0.013
	11	1.26	0.003	0.019
	12	0.85	0.002	0.009
	13	1.18	0.003	0.015
	14	0.28	0.001	0.001
	15	0.82	0.003	0.010
Lake Erie	1	0.70	0.002	0.012
prior to ovulation	2	0.78	0.001	0.011
	3	1.18	0.001	0.019
	4	0.39	0.001	0.008

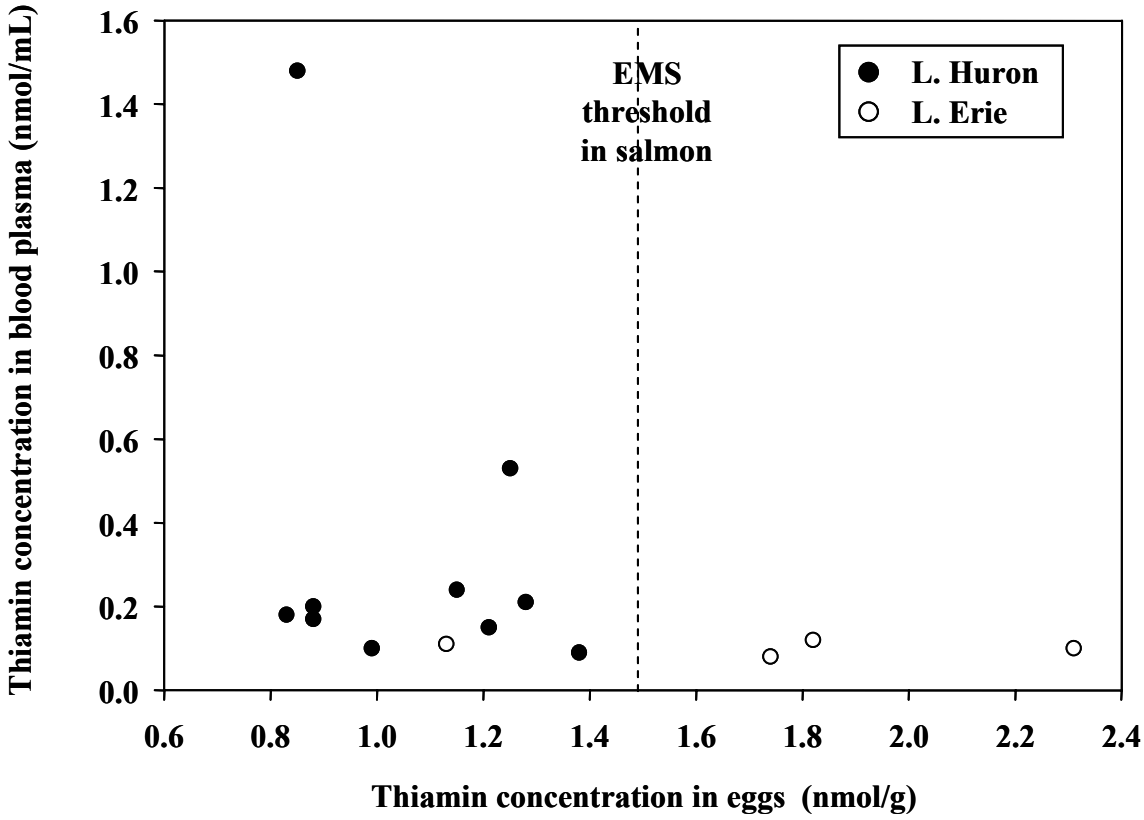

Figure 1: Relationship between concentration of thiamin in blood plasma and eggs from individual lamprey females (freshwater origin). The threshold for landlocked Atlantic salmon was arbitrally chosen based on data from Figure 5 in Fisher et al. (1998).

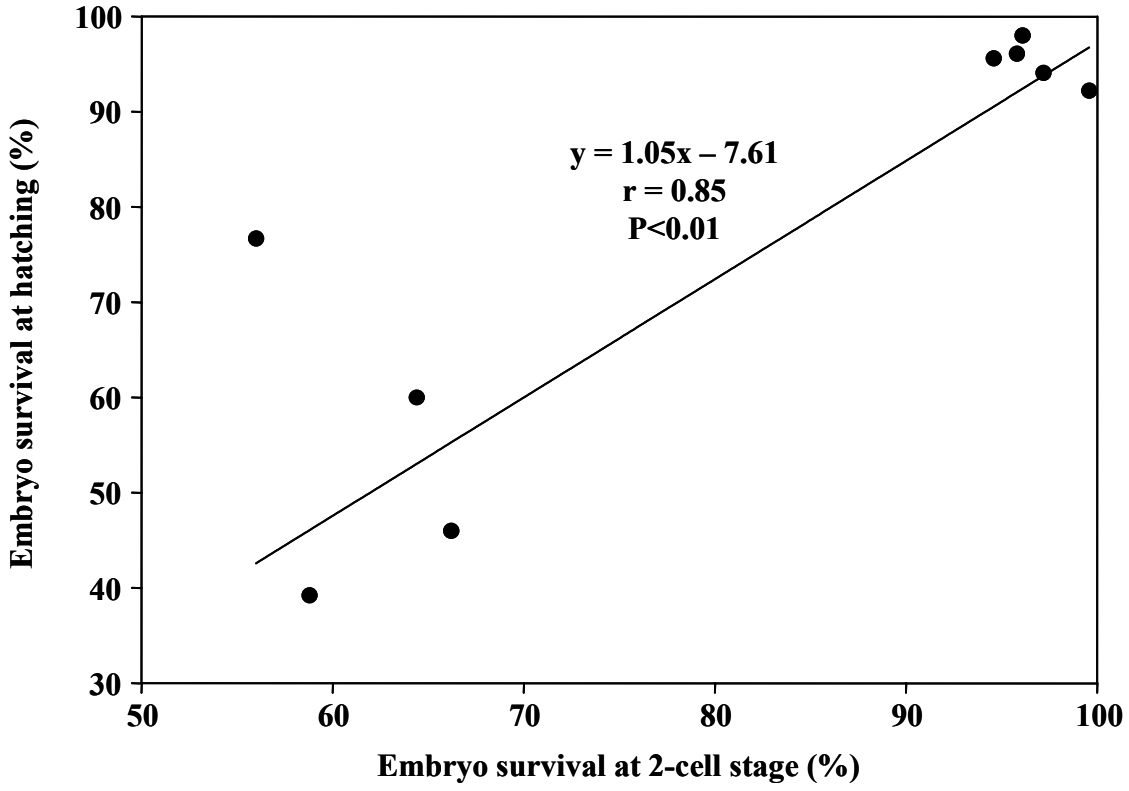

Figure 2: Regression between percentage of 2-cell embryos and hatched larvae for individual females from L. Huron.

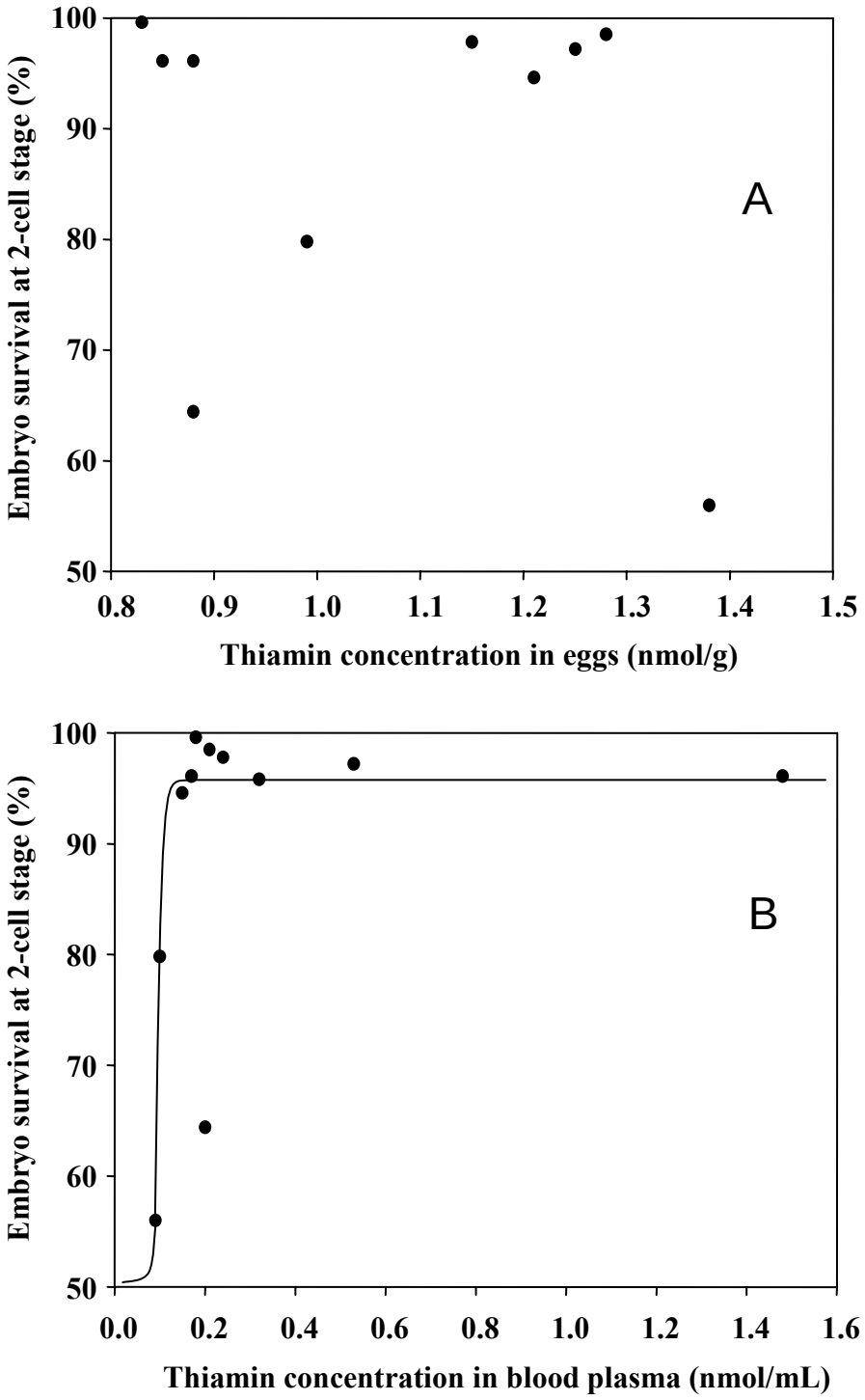

Figure 3: Thiamin levels in eggs (A) or blood plasma (B) of individual lamprey females and survival of embryos.

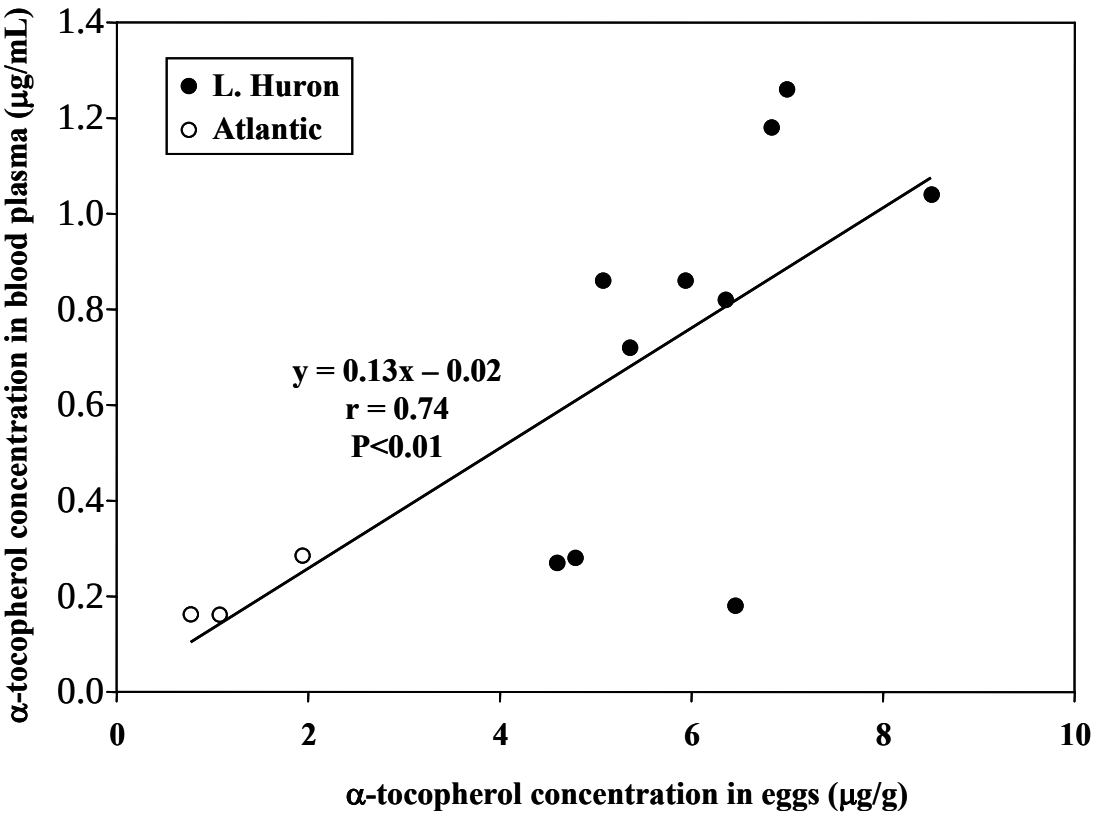

Figure 4: Correlation between vitamin E concentrations in tissues from individual females from L. Huron and Atlantic.

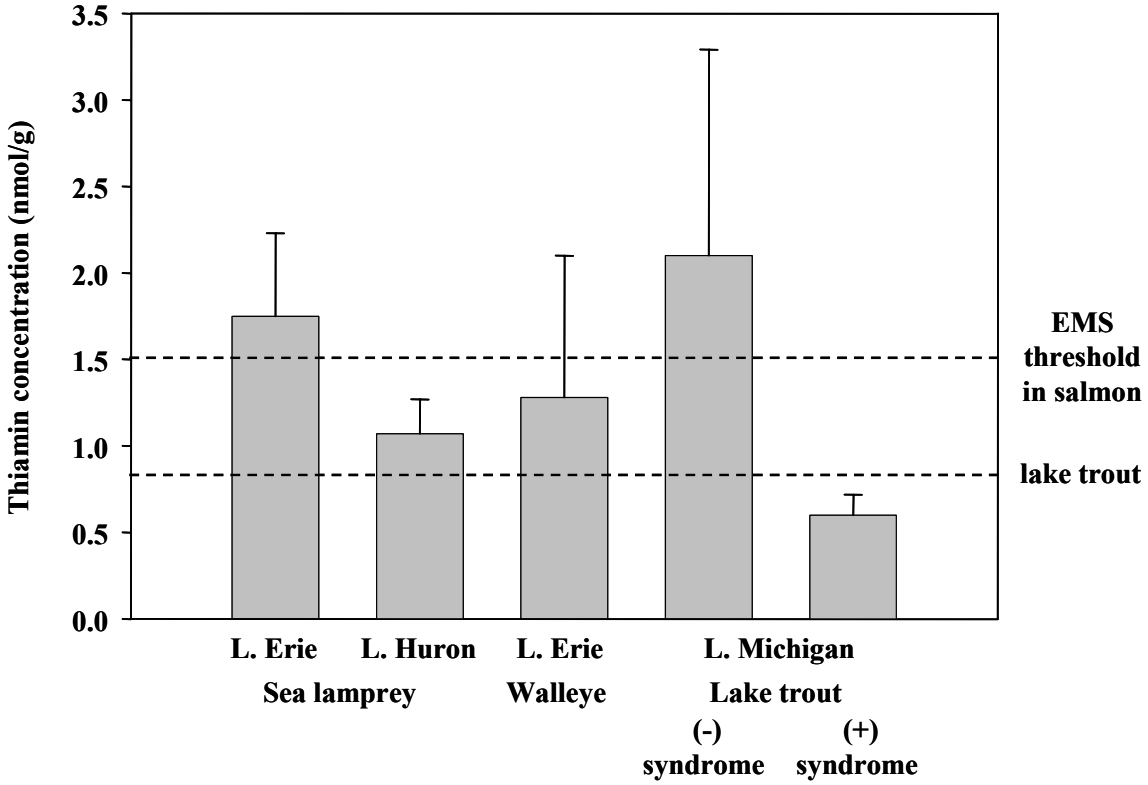

Figure 5: Mean thiamin levels in eggs from lamprey and two teleost fish. Data for L. Erie's walleye (n=21) were collected at the same year (2003) (Rinchard and Dabrowski, unpublished). Data for lake trout (n=19) includes fish that produce progenies with high mortalities (>42%) and EMS syndrome and fish with high survival until first exogenous feeding ((-)-syndrome) (Czesny, Dettmers, Rinchard and Dabrowski, unpublished). "EMS thresholds" refer to thiamin concentrations that resulted in high mortalities in landlocked salmon (Fig. 5 in Fisher et al., 1998) and lake trout (Fitzsimons et al., 1999).