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[Article]

Molecular Markers of Yolk Sac Fry Development in Nine Families of Lake Trout

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Abstract.—Salmonids in certain areas of North America and northern Europe suffer from reproductive disturbances manifested through the death of yolk sac fry. These disturbances are referred to as early mortality syndrome (EMS) in the Great Lakes region and M74 in the Baltic Sea. Both of these syndromes have been associated with reduced concentrations of thiamine in affected females and their eggs. However, large variations in signs and mortality, both within and between the individual syndromes, have been reported. Yolk sac fry mortality (M74) in Atlantic salmon *Salmo salar* has been shown to be associated with reduced DNA binding of the hypoxia-inducible transcription factor 1 (HIF-1), reduced production of vascular endothelial growth factor (VEGF) protein, decreased capillary density, and down-regulation of adult-type globin gene transcription (which is responsible for the protein part of adult hemoglobin). One of the main effects of all of these changes is reduced oxygen transport to the tissues of affected fry. In this study, the developmental patterns of HIF-1 DNA binding, VEGF protein expression, and adult-type globin gene transcription were analyzed in nine family groups of Lake Michigan lake trout *Salvelinus namaycush*. The results indicate that HIF-1 DNA binding and globin gene transcription increase from hatch to the end of yolk sac stage. Interindividual and between-family biological variations were detected, especially in VEGF protein expression and globin gene transcription. Our results demonstrate the possibility of using these molecular markers in investigating the etiology of EMS and making comparisons between the mechanisms of different salmonid yolk sac fry mortalities.

Early life stage mortalities in salmonids have been described in the Great Lakes of North America, New York's Finger Lakes, and the Baltic Sea. There are striking similarities between early mortality syndrome (EMS) in North America and M74 in the Baltic Sea basin with respect to the timing of onset and the clinical signs of affected individuals. These similarities are remarkable given the geographic separation of the affected areas and pose intriguing questions relating to the underlying molecular mechanisms responsible for triggering these diseases. Both of these syndromes have recently been a research focus owing to concerns about conservation and the self-sustainability of the affected stocks.

The early life stage mortality of salmonids with clinical signs resembling EMS is called Cayuga syndrome in Atlantic salmon *Salmo salar* from the Finger Lakes (Fisher et al. 1995) and M74 in those from the Baltic Sea (Bengtsson et al. 1999; Vuori and Nikinmaa 2007). These early life stage mortality syndromes have been shown to be associated with reduced concentrations of thiamine in affected females and their eggs (Fisher et al. 1995; Amcoff et al. 1999; Fitzsimons et al. 1999; Honeyfield et al. 1999). However, large variations in clinical signs and mortality, both within and between the individual syndromes, have been reported. Generally, EMS is characterized by the partial mortality of affected family groups before the swim-up stage, whereas M74 is often associated with the complete mortality of affected family groups.

During salmonid yolk sac fry development, new capillaries are formed and larval-type red blood cells and hemoglobins are replaced by adult types (Iuchi and Yamamoto 1983; Wells and Pinder 1996a). As some of the signs of EMS, M74, and Cayuga syndrome (Fitzsimons et al. 1999), such as decreased heart rate and blood congestion, are cardiovascular, yolk sac fry

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mortality may relate to oxygen transport. Notably, in M74-affected Atlantic salmon yolk sac fry from the Baltic Sea a group of signs associated with the development and maintenance of the vasculature and circulation has been described, including impaired vascular development, a reduced number of circulating erythrocytes, and abnormal hemorrhages or blood coagulation (Norrgren et al. 1993; Lundstrom et al. 1998, 1999).

Cardiovascular defects are observed in mammalian embryos when the function of the transcription factor (hypoxia-inducible transcription factor 1α [HIF-1α]), its dimerization partner (aryl hydrocarbon nuclear translocator [ARNT]), or target gene (vascular endothelial growth factor [VEGF]), is disturbed (Carmeliet et al. 1996; Maltepe et al. 1997; Ryan et al. 1998). HIF-1α is a member of the bHLH-PAS transcription factors, which regulate environmentally induced and developmentally controlled gene expression (Crews and Fan 1999; Gu et al. 2000). Specifically, HIF-1α is an important regulator of hypoxia responses, including vascularization and erythropoiesis (Semenza 2004; Nikinmaa and Rees 2005; Wenger et al. 2005), and is required for normal development, including angiogenetic, hematopoietic, and neural development in mammalian embryos (Ryan et al. 1998). Vuori et al. (2004) have established that the intermediate type of progression of M74 (death of yolk sac fry at 180–190 degree-days after hatch) is associated with reduced DNA binding of HIF-1α, reduced production of VEGF protein, and decreased capillary density. These results, together with the down-regulation of adult-type hemoglobin gene transcription (Vuori et al. 2006), indicate that M74-affected fry suffer from internal hypoxia at the time that they shift from skin- to gill-breathing and change from the production of larval- to adult-type hemoglobins and red blood cells.

The aim of this study was to analyze the developmental patterns of HIF-1 DNA binding, VEGF protein expression, and adult-type globin gene transcription from family groups of lake trout *Salvelinus namaycush* originating from southwestern Lake Michigan (an area where EMS is commonly observed) and to discuss the possibility of using these molecular markers in comparing the etiologies of EMS and M74.

Methods

Lake trout gamete collection, fertilization, incubation, and sampling.—Lake trout were captured in gill nets set by the Illinois Department of Natural Resources during the fall 2004 population assessment in southwestern Lake Michigan. Fish were caught on the Waukegan Reef (6 mi east of Waukegan Harbor [1 mi = 1.61 km]). Ripe females ($n = 9$) were stripped of eggs. A subsample (5 g) of the eggs from each female was immediately frozen on dry ice and stored at −80°C until thiamine analysis. Sperm was obtained from several males and stored on ice. Unfertilized eggs and sperm were transported to the Illinois Natural History Survey's Lake Michigan Biological Station. Upon arrival, sperm from individual males was checked for motility, and only motile semen was used for fertilization. The eggs from each female (~100 eggs) were fertilized in three replicates with 100 μL of pooled (five males) 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, Tacoma, Washington) supplied with flow-through Lake Michigan water. Temperature was monitored several times a day.

To assess EMS frequency, newly hatched embryos from each family group with no signs of abnormalities were placed in 30-L aquaria (~100 individuals/aquarium) supplied with flow-through lake water at ambient temperature (4–5°C) and continuous aeration. Temperature was measured three times a day, and fish were monitored for signs of EMS (lethargy, loss of equilibrium, and spiral and erratic swimming). During embryonic and posthatch development, relative age was followed in terms of cumulative degree-days (CDD, i.e., the mean daily temperature [°C] multiplied by the number of days postfertilization). The experiment was terminated at 1,000 CDD and the EMS frequency in each family group quantified.

A separate set of newly hatched embryos (~200 individuals) from corresponding family groups was established in a series of 30-L aquaria and used for protein and RNA sampling. The rearing conditions were identical to those described above. Lake trout yolk sac fry were sampled at eight consecutive points of development (0, 50, 100, 150, 200, 250, 300, 350 CDD posthatch). For RNA analysis, three individuals were preserved in RNA*later* solution (Ambion, Austin, Texas) at each age point following all standard precautions related to RNA contamination risks. For protein analysis, five individuals were sampled and immediately frozen at each age period. All samples were stored below −80°C, and once the experiment was concluded samples were shipped on dry ice to the University of Turku, Finland, for RNA and protein analysis.

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 chromatography (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, 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 fluorescent 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 (85 mM; pH 7.5) plus acetonitrile (65:35 by volume). The flow rate was 0.5 mL per min. The column thermostat was set to 30°C. Each external standard curve for TH, TMP, and TPP was prepared 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, 101.1 ± 3.1% ($n = 4$) for TMP, and 103.2 ± 3.8% ($n = 4$) for TPP. For the recovery, known amounts of TH, TMP, and TPP standards were added to running samples at the beginning of the extraction and followed by the extraction procedure as described above.

Sample preparation.—Whole yolk sac fry nuclear protein extracts were prepared according to the method of Semenza and Wang (1992), with the following modifications: a whole fry was homogenized with dounce homogenizer in buffer A, and the protease inhibitors used were 0.5 mM phenylmethylsulphonyl fluoride, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, 2 μg/mL approtinin, 2 μg/mL leupeptin, 2 μg/mL antipain, and 2 μg/mL pepstatin. Protein concentrations were measured with Biorad's Bradford protein assay reagent according to the manufacturer's protocol. Total RNA was isolated from whole yolk sac fry using TRI-reagent (Sigma-Aldrich, St. Louis, Missouri) according to the manufacturer's instructions. An additional purification of the extracted RNA was performed using RNeasy (Qiagen, Germantown, Maryland) according to the manufacturer's instructions. The quality of RNA was monitored using the 260:280 nm optical density ratio and agarose gel. Complementary DNA (cDNA) was prepared from total RNA (3 μg) using Powerscript reverse transcriptase (BD Biosciences, Erembodegem, Belgium) and oligo(dT)12–18 primer (Invitrogen, Carlsbad, California) for real-time polymerase chain reaction (PCR) samples and 3′CDS primer (BD Biosciences) for 3′ rapid amplification of cDNA ends (RACE) cloning according to the manufacturers' instructions.

Electrophoretic mobility shift assay and Western blotting.—An electrophoretic mobility shift assay (EMSA) was performed as described by Vuori et al. (2004) using 10 μg of nuclear extract and 0.1 μg of poly (deoxyinosinic-deoxycytidylic) acid (Sigma-Aldrich) as a carrier. The HIF-1 binding site (hypoxia response element) in the promoter region of the human erythropoietin gene was used as a probe (Kvietikova et al. 1995). It has been ascertained that this probe can also be used on fish (Soitamo et al. 2001). Western blotting for HIF-1α and VEGF165 protein was conducted as described in Vuori et al. (2004). The density values of the protein bands were determined from autoradiography film using a Chemi-Imager digital camera and software (Alpha Innotech Co., San Leandro, California).

3′ RACE cloning and real-time PCR quantification of lake trout globins.—Cloning of the 3′ ends of lake trout adult-type hemoglobin subunit alpha-4 (HBAIV) and beta globin (HBB) mRNA was conducted with the SMART RACE cDNA amplification kit (Clontech, Mountain View, California) as recommended in the manufacturer's instructions. The two primers used for the 3′ RACE cloning of HBAIV were HBAIV_3RACE1_5′-ACAAAGCCAACGTGAAGGCCATCTG-3′ and HBAIV_3RACE2_5′-GTGAAGAAGCACGGCATCACCATCA-3′, and the two primers used for the 3′ RACE cloning of HBB were HBB_3RACE2_5′-GTGGGGAAAGATCAGCGTGGATGAG-3′ and HBB_3RACE3_5′-CCATCGTAGGCCTGTGGGGAAAGAT-3′.

The PCR products were transferred and run in agarose gels, the bands excised and extracted with a Montage DNA gel extraction kit (Millipore, Billerica, Massachusetts), and the fragments sequenced. The complete cloned sequences are shown in an appendix and the alignments with known salmonid HBAIV and HBB mRNA sequences in Figure 1. The PCR primers and TaqMan probes for HBAIV and HBB were obtained from Applied Biosystems's Custom TaqMan gene expression assay service. TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California) was used for real-time PCR quantification of HBAIV and HBB. The copy number of each sample was standardized to the 60S ribosomal L32 gene as described in Vuori et al. (2006). Relative standards of Ultra FreeDA (Millipore) purified PCR products of known relative dilutions were made for each transcript.

Statistics.—SPSS 12.0.1. software was used for statistical analyses. A repeated-measurements general linear model (GLM) with least-squares differences was used as the post hoc test for HIF-1 DNA-binding, VEGF protein, and hemoglobin transcription age series analysis. For the initial 50- and 150-CDD HIF-1 DNA-binding and VEGF protein analysis, analysis of variance (ANOVA) with least-squares differences was used. However, to simplify the visualization of

FIGURE 1.—**(a)** Lake trout HBAIV mRNA sequences obtained with 3′ RACE cloning (see Methods) aligned with Atlantic salmon hemoglobin subunit alpha-4 (accession number BT058900) and rainbow trout alpha-globin I (accession number D88113) mRNA sequences and **(b)** lake trout HBB mRNA sequences obtained with 3′ RACE cloning aligned with Atlantic salmon beta-globin (accession number X69958) and rainbow trout beta-globin (accession number D82926) mRNA sequences. The sequences were aligned using the CLUSTAL 2.0.10 multiple-sequence alignment tool. Stars indicate similar nucleotides in all four sequences.

these differences, two-step clustering of similar family groups was used instead of showing the individual between-group statistical differences. Linear regression was used to evaluate possible thiamine-dependent effects on the molecular markers. The group- and age-specific numbers of specimens used in the HIF-1 DNA-binding (EMSA), VEGF protein, and HBA and HBB transcription analyses are shown in Table 1. Differences were considered statistically significant at $\alpha < 0.05$.

Results

The thiamine levels in the eggs of the nine monitored lake trout families and the frequency of mortality from EMS and yolk edema are summarized in Table 2. Two of the nine groups (2 and 9) had egg thiamine levels below 0.8 nmol/g, the threshold at which increased frequency of EMS was reported in lake trout from Lake Ontario (Brown et al. 1998a), while the remaining groups had relatively high thiamine levels. Neither yolk edema nor EMS mortality was correlated with the thiamine concentration in the eggs.

Initially, HIF-1α protein and DNA-binding levels were studied at eight different age points for lake trout yolk sac fry development (0, 50, 100, 150, 200, 250, 300, and 350 CDD). We found that the expression of HIF-1α protein and HIF-1 DNA binding to the hypoxia response element increases with age, as shown in the representative Western blot and EMSA autoradiography films in Figure 2. HIF protein is undetectable at 0 and 50 CDD and hardly visible at 100 CDD. There is, however, some detectable DNA binding at these ages. Figure 3a shows the HIF DNA-binding pattern from 0 to 300 CDD in six different family groups and that from 50 and 150 CDD in nine different family groups. The age series of HIF-1 DNA-binding pattern from 0 to 300 CDD is

b

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D82926_OM        TGAGCGCAGTGCCATCGTAGGCCTGTGGGGAAAGATCAGCGTGGATGAGATCGGACCCCA 88
X69958_SS        TGAGCGCAGCGCCATCGTAGGCCTGTGGGGAAAGATCAGCGTGGATGAGATCGGACCCCA 119
HBB_RACE2_SN     TA---GTGATT-------------GTGGGGAAAGATCAGCGTGGATGAGATCGGACCCCA 102
HBB_RACE3_SN     TA---GTGATTCCATCGTAGGCCTGTGGGGAAAGATCAGCGTGGATGAGATCGGACCCCA 113
                 *    *                  ************************************

D82926_OM        GGCCCTGGCCAGACTTCTGATCGTGTCTCCATGGACTCAGAGACACTTCAGCACCTTCGG 148
X69958_SS        GGCCCTGGCCAGACTTCTGATCGTGTCTCCATGGACTCAGAGGCACTTTAGCACCTTCGG 179
HBB_RACE2_SN     GGCCCTGGCCAGACTTCTGATCGTGTCTCCATGGACTCAGAGACACTTCAGCACCTTCGG 162
HBB_RACE3_SN     GGCCCTGGCCAGACTTCTGATCGTGTCTCCATGGACTCAGAGACACTTCAGCACCTTCGG 173
                 ****************************************** ***** ***********

D82926_OM        CAACCTGTCCACACCCGCTGCCATCATGGGTAACCCCGCCGTGGCCAAGCACGGAAAGAC 208
X69958_SS        CAACCTGTCCACACCCGCTGCCATCATGGGTAACCCCGCCGTGGCCAAGCACGGAAAGAC 239
HBB_RACE2_SN     CAACCTGTCCACACCCGCTGCCATCATGGGTAACCCCGCCGTGGCCAAGCACGGAAAGAC 222
HBB_RACE3_SN     CAACCTGTCCACACCCGCTGCCATCATGGGTAACCCCGCCGTGGCCAAGCACGGAAAGAC 233
                 ************************************************************

D82926_OM        CGTGATGCACGGACTGGACAGAGCTGTGCAGAACCTGGATGACATCAAGAACACCTATAC 268
X69958_SS        CGTGATGCACGGACTGGACAGAGCTGTGCAGAACCTGGATGACATCAAAAACGCCTATAC 299
HBB_RACE2_SN     CGTGATGCACGGACTGGACAGAGCTGTGCAGAACCTGGATGACATCAAGAACACCTATAC 282
HBB_RACE3_SN     CGTGATGCACGGACTGGACAGAGCTGTGCAGAACCTGGATGACATCAAGAACACCTATAC 293
                 ********************************************** *** *******

D82926_OM        TGCACTGAGTGTGATGCACTCCGAGAAACTGCACGTGGATCCCGACAACTTCAGGCTCCT 328
X69958_SS        TGCCCTGAGTGTGATGCACTCCGAGAAACTGCACGTGGATCCCGACAACTTCAGGCTCCT 359
HBB_RACE2_SN     TGCACTGAGTGTGATGCACTCCGAGAAACTGCACGTGGATCCCGACAACTTCAGGCTCCT 342
HBB_RACE3_SN     TGCACTGAGTGTGATGCACTCCGAGAAACTGCACGTGGATCCCGACAACTTCAGGCTCCT 353
                 *** ************************************************************

D82926_OM        TGCCGACTGCATCACCGTGTGCGTGGCCGCCAAGCTCGGTCCCGCCGTTTTCAGTGCTGA 388
X69958_SS        CGCCGACTGCATCACCGTGTGCGTGGCAGCCAAGCTCGGTCCCACCGTTTTCAGTGCTGA 419
HBB_RACE2_SN     CGCCGACTGCATCACCGTGTGCGTGGCCGCCAAGCTCGGTCCCGCCGTTTTCAGTGCTGA 402
HBB_RACE3_SN     TGCCGACTGCATCACCGTGTGCGTGGCGGCCAAGCTCGGTCCCACCGCTTTCAGTGCTGA 413
                  ************************** *************** *** ************

D82926_OM        TACTCAGGAAGCCTTCCAGAAGTTCCTGGCTGTCGTTGTGTCCGCTCTTGGCAGACAGTA 448
X69958_SS        TATTCAGGAAGCCTTCCAGAAGTTCCTGGCTGTCGTTGTGTCCGCTCTTGGCAGACAGTA 479
HBB_RACE2_SN     TACTCAGGAAGCCTTCCAGAAGTTCCTGGCTGTCGTTGTGTCCGCTCTTGGCAGACAGTA 462
HBB_RACE3_SN     TACTCAGGAAGCCTTCCAGAAGTTCCTGGCTGTCGTTGTGTCCGCTCTTGGCAGACAGTA 473
                 ** ************************************************************

D82926_OM        CCACTAGAGCATCACTCGACAGCATCAATATGGAAGAGAGATGACACTGACACTCCAACT 508
X69958_SS        CCACTAGAGCATCACTCGACAGCATCAATATGGAAGAGAGATGACACT------CCAA-T 532
HBB_RACE2_SN     CCACTAGAGCATCACTCGACAGCATCAATATGGAAGAGAGATGACACT------CCAACT 516
HBB_RACE3_SN     CCACTAGAGCATCACTCGACAGCATCAATATGGAAGAGAGATGACACT------CCAACT 527
                 ************************************************      **** *

D82926_OM        CCAGTCTGTTAGGCTGGAAGCTG-GC---------------------------------- 533
X69958_SS        CCAGTCTGTTAGGCTGGAAGCTGTGCCATAGCTACACAT-TTGAAAAAAATTAAATCTAT 591
HBB_RACE2_SN     CCAGTCTGTTAGGCTGGAAGCTGTGCCATAGCTACACATATTGAAAATAATAAAATACAT 576
HBB_RACE3_SN     CCAGTCTGTTAGGCTGGAAGCTGTGCCATAGCTACACAT--TTAAAAAAATAAAATCAAT 585
                 ********************** **
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FIGURE 1.—Continued.

similar in family groups 3, 7, 8, and 9. The DNA-binding pattern from 0 to 300 CDD in family group 2 differs significantly from that of the other groups, and the pattern in family group 1 differs significantly from those of the rest of the groups except for group 8 (GLM). At 50 and 150 CDD, HIF-1 DNA binding was analyzed for nine different family groups; Figure 3b shows the similarities and differences between these groups. In general, we did not find any clear thiamine-dependent effect on HIF-1 DNA binding.

Figure 4a shows the VEGF protein expression pattern from 0 to 300 CDD in six different family groups and from 50 and 150 CDD in nine different family groups. The age series from 0 to 300 CDD is similar in family groups 1 and 3 and groups 7, 8, and 9, whereas that of group 2 differs significantly from those of the other groups (GLM). At 50 and 150 CDD, VEGF protein expression was analyzed for nine different family groups; Figure 4b shows the similarities and differences between these groups. At 150 CDD, lower levels of VEGF protein were detected in groups having the lowest total thiamine concentrations (2 and 9) as well as in group 8. These differences were statistically significant according to the linear regression and ANOVA. No other thiamine-dependent effects were observed.

TABLE 1.—Group- and age-specific numbers of specimens used in the HIF-1 DNA-binding (EMSA), vascular endothelial growth factor (VEGF) protein, adult-type hemoglobin (HBA), and beta-globin (HBB) transcription analyses.

Group	0	50	100	150	200	250	300	350
				EMSA				
1	2	5	4	4	5	5	2	
2	5	5	5	6	5	3	5	
3	2	5	5	3	5			
4		5		5				
5		5		5				
6		5		5				
7	6	5	6	5	5	2	3	
8	5	5	5	5	5			
9	5	5	5	5	5	3		
				VEGF				
1	2	5	3	5	5	5	3	
2	4	6	5	6	5	4	5	
3	2	5	4	3	5			
4		5		5				
5		5		5				
6		5		5				
7	4	5	6	4	5	2	3	
8	4	5	2	5	5			
9	4	5	4	5	5	3		
				HBA				
1	2		3		3		3	
2	3		3		3		3	3
3	3		3		3			
4								
5								
6								
7	5		6		6			3
8	2		3		3			
9	3		3		3		3	
				HBB				
1	2		3		3		3	
2	3		3		3		3	3
3	3		3		3			
4								
5								
6								
7	5		6		6			3
8	2		3		3			
9	3		3		3		3	

The age series of HBA and HBB transcription from 0 to 350 CDD was analyzed from six family groups (Figure 5). We found that the transcription of HBA and HBB increases with age. The age series of HBA transcription is significantly different in family group 2 than in the other groups (GLM). The age series of HBB transcription is significantly different in family group 9 than in groups 1, 2, and 7. In general, we did not find any clear thiamine-dependent effect on HBA and HBB transcription.

Discussion

The offspring of the nine lake trout family groups considered in this study generally had a low frequency of EMS-related mortality, even in the groups with egg free thiamine levels below 0.8 nmol/g. Below this value, EMS frequency becomes highly variable but correlates with the free thiamine level in the eggs (Brown et al. 1998a; Czesny et al. 2009, this issue). The thiamine levels in studied groups were relatively low but consistent with those for other years in southwestern Lake Michigan (Czesny et al. 2009).

HIF-1α is a master regulator of both developmental and adaptive hypoxia responses. Vertebrate HIFs are known to participate in several developmental processes, including angiogenesis, vasculogenesis, and heart and central nervous system development (Ryan et al. 1998; Sipe et al. 2004; Rojas et al. 2007). Angiogenesis and erythropoiesis also occur as physiological responses to hypoxic exposure (Semenza 2004; Nikinmaa and Rees 2005; Wenger et al. 2005). On the basis of the present results, lake trout yolk sac fry have an increasing expression pattern of HIF-1α protein and HIF-1 DNA binding from 0 to 350 CDD after hatch. Such patterns have also been reported in association with Atlantic salmon yolk sac fry development after hatch (Vuori et al. 2004) and in African clawed frog *Xenopus laevis* tadpoles (Sipe et al. 2004). During salmonid yolk sac fry development, new capillaries are

TABLE 2.—Concentrations (mean ± SD) of free thiamine (TH), thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and total thiamine in lake trout eggs and embryo mortality at different stages of development.

	Thiamine concentration (nmol/g)				Mortality (%)	
Female	TH	TMP	TPP	Total	Yolk edema–blue sac	Early mortality syndrome
1	8.05 ± 6.12	0.24 ± 0.18	0.49 ± 0.57	8.78 ± 6.87	2.7	5.3
2	0.55 ± 0.03	0.12 ± 0.01	0.35 ± 0.02	1.02 ± 0.08	0.0	5.5
3	3.01 ± 0.72	0.09 ± 0.00	0.06 ± 0.03	3.16 ± 0.75	1.4	8.1
4	4.46 ± 1.16	0.24 ± 0.09	0.44 ± 0.15	5.14 ± 1.40	6.8	3.0
5	4.18 ± 0.52	0.33 ± 0.09	0.69 ± 0.11	5.20 ± 0.31	2.7	5.3
6	3.88 ± 1.34	0.20 ± 0.11	0.35 ± 0.36	4.43 ± 1.81	9.3	2.3
7	4.20 ± 2.71	0.21 ± 0.14	0.51 ± 0.46	4.92 ± 3.30	8.8	0.6
8	3.13 ± 0.12	0.14 ± 0.03	0.08 ± 0.00	3.36 ± 0.16	4.3	5.5
9	0.78 ± 0.01	0.09 ± 0.00	0.08 ± 0.01	0.96 ± 0.01	2.9	4.1

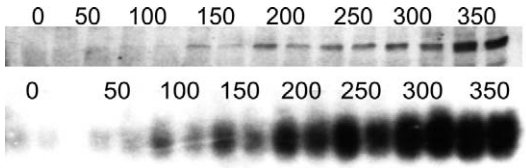

FIGURE 2.—Representative Western blot and EMSA autoradiography films of HIF-1α protein expression and HIF-1 DNA binding in two lake trout yolk sac fry at 0–350 CDD after hatch. In the HIF protein series there is only one 0-CDD specimen.

formed, gill lamellae and filaments develop, and larval-type red blood cells and hemoglobins are replaced by adult types (Iuchi and Yamamoto 1983; Wells and Pinder 1996a). These events, reflecting the change from cutaneous to primarily branchial respiration (Wells and Pinder 1996a, 1996b), could be at least partly regulated by HIF-1α. The reasons for the increased activity of HIF-1 are not known at present.

VEGF is involved in every stage of vertebrate vascular development (Nasevicius et al. 2000; Ruhrberg 2003). The assembly of vessels from precursor cells (vasculogenesis), the modification of this primary vasculature (angiogenesis) and the recruitment and differentiation of vessels into target tissues according to their specific needs are all regulated by VEGF (Ruhrberg 2003). Loss of a single VEGF allele in mouse models leads to gross developmental deformities in the forming vasculature and embryonic death (Carmeliet et al. 1996; Ferrara et al. 1996). VEGF is

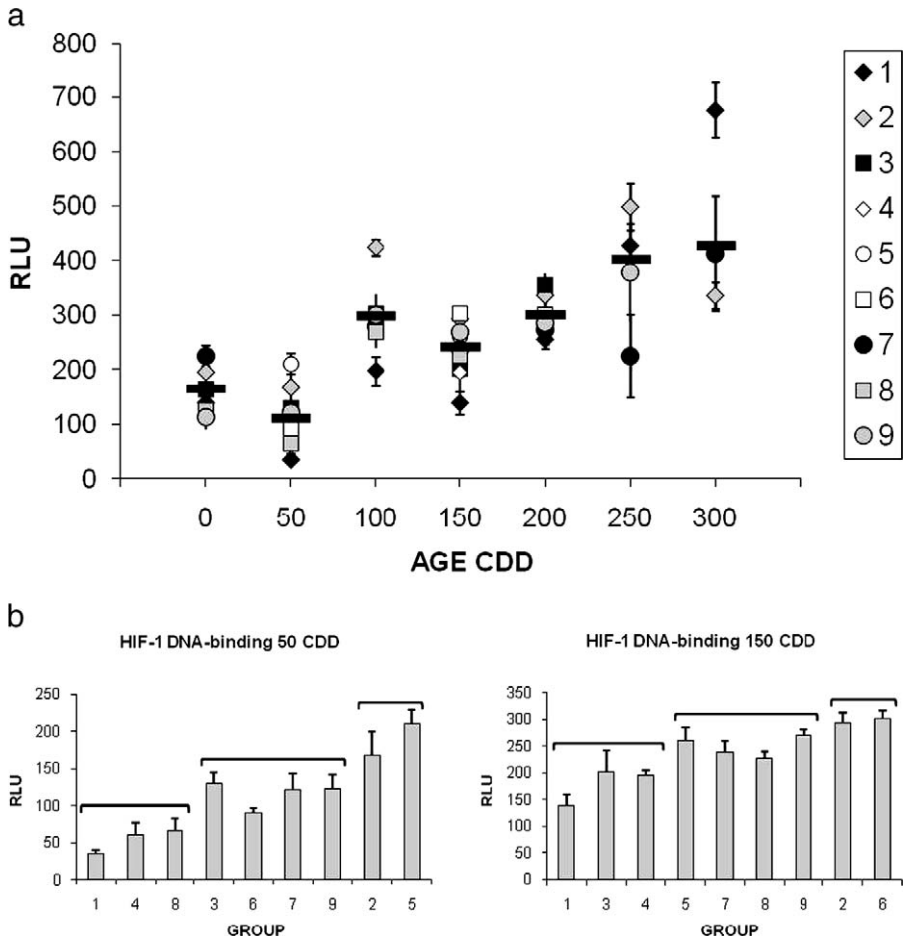

FIGURE 3.—Panel **(a)** shows the HIF-1 DNA-binding patterns from 0 to 300 CDD after hatch in six lake trout yolk sac fry family groups (1, 2, 3, 7, 8, and 9) and those at 50 and 150 CDD in all nine lake trout family groups (see Table 2). The dark horizontal lines represent means for all of the family groups included; RLU = relative light units. Panel **(b)** shows the HIF-1 DNA binding at 50 and 150 CDD. Clusters of similar family groups (based on two-step clustering) are indicated by brackets. The error bars represent standard errors of the means.

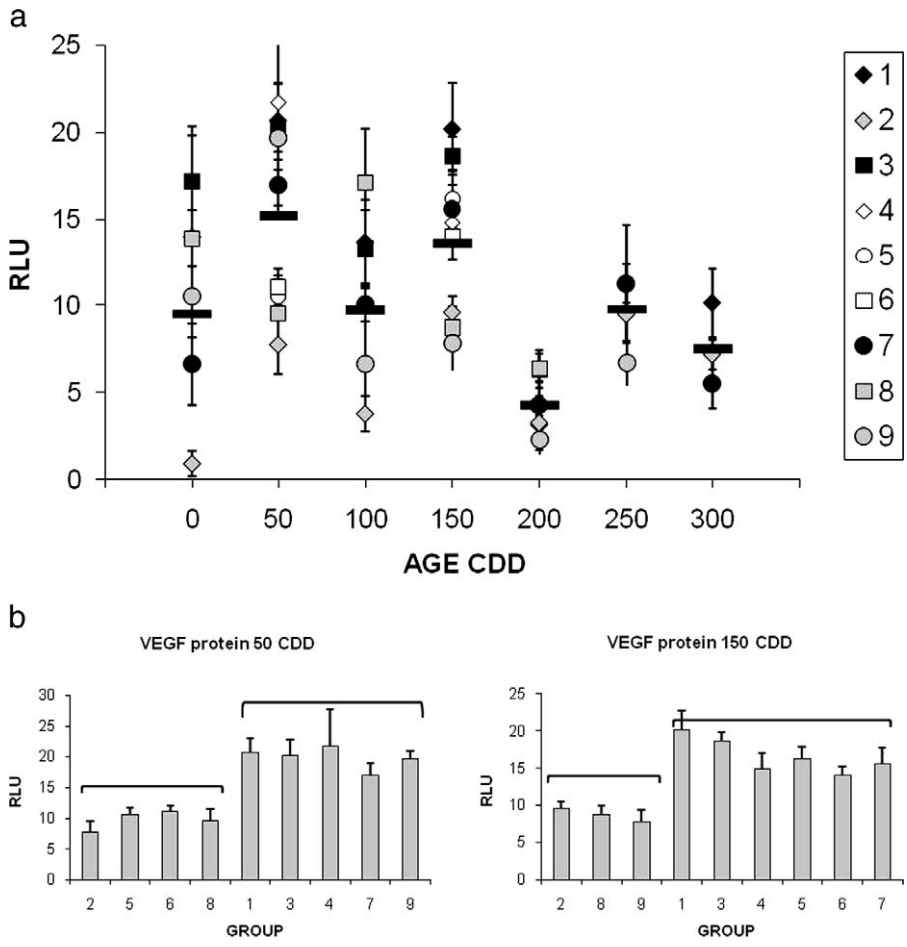

FIGURE 4.—Panel **(a)** shows the VEGF protein expression patterns from 0 to 300 CDD after hatch in six lake trout yolk sac fry family groups (1, 2, 3, 7, 8, and 9) and those at 50 and 150 CDD in nine lake trout family groups; panel **(b)** shows the VEGF protein expression at 50 and 150 CDD. See Figure 3 for additional details.

thus a major growth factor involved in the formation of new capillaries and may be involved in the production of an optimal capillary network when the fish fry grow and the skin–gill change in the primary respiratory surface occurs. In fish, reduced VEGF levels may influence blood cell formation (Liang et al. 2001) in addition to causing severe defects in vascular development. Previous results (Vuori et al. 2004) indicate reduced VEGF protein expression in M74-affected Atlantic salmon. The effect of EMS on VEGF protein expression levels could not be addressed in this study, as the groups considered exhibited a low frequency of EMS. Interindividual and between-family biological variation in VEGF protein expression was detected, especially from 0 to 150 CDD after hatch. The variation was not, however, clearly associated with thiamine levels.

In salmonids, the period of hemoglobin (and red blood cell) switching from embryonal to adult types occurs after hatching of the yolk sac fry (Iuchi and Yamamoto 1983). The transcription of the adult globin genes increases between 50 and 180 CDD in normal Atlantic salmon yolk sac fry development. This increase is blunted during the development of M74-affected fry, and these fry suffer from clear down-regulation of adult-type hemoglobin transcription from the preclinical to the terminal stages of the disease (Vuori et al. 2006). The decrease in hemoglobin transcription in M74 family groups is independent of the time when mortality starts. Based on our results, it appears that adult-type hemoglobin (HBA and HBB) transcription in lake trout yolk sac fry increases with age. The increase of HBA and HBB transcription is associated with an increase in HIF-1 DNA binding in both lake trout and Atlantic

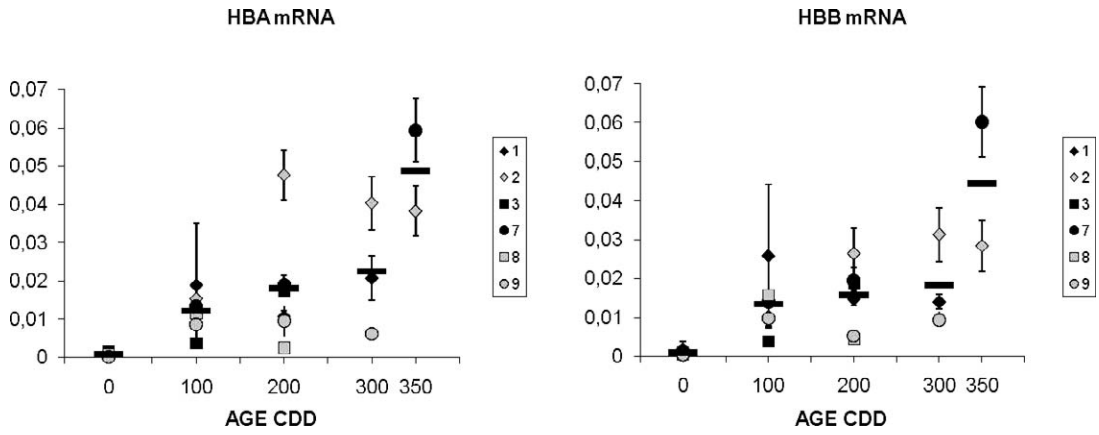

FIGURE 5.—HBA and HBB mRNA transcription from 0 to 350 CDD after hatch in six lake trout yolk sac fry family groups (see Table 2). The values along the *y*-axes are copy numbers standardized to the 60S ribosomal L32 gene. The dark horizontal lines represent means for all of the family groups included; the error bars represent standard errors of the means.

salmon, and this may represent a common developmental pattern in salmonids.

In this study, low-thiamine groups that were affected by EMS showed only partial mortality (<10%), and the specimens analyzed were expected to be among the survivors. Therefore, no clear connection could be established between the etiology of EMS and the molecular markers analyzed. Low-thiamine-group 2 did have some anomalies in HIF-1 DNA binding, VEGF protein expression, and HBA and HBB transcription patterns compared with the other groups. However, the changes observed in group 2 were not uniform with those in the other low-thiamine group (9), so we cannot draw any firm conclusions as to the effects of low thiamine levels on the molecular markers we studied.

In summary, we found that mean HIF-1 DNA binding and globin gene transcription of nine lake trout family groups increased from hatch to the end of yolk sac stage. Interindividual and between-family biological variations were detected, especially in VEGF protein expression at 0–150 CDD and in globin gene transcription. These results demonstrate that the molecular markers analyzed may be used to study the etiology of EMS in lake trout. These markers could be used to compare families experiencing EMS with families from an area without EMS and in further investigations of the molecular background of the signs in different yolk sac fry mortalities.

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Appendix: Complete Cloned Sequences of the 3′ Ends of Lake Trout Adult-Type Hemoglobin Subunit Alpha-4 (HBAIV) and Beta Globin (HBB) mRNA.

>HBaIV_RACE1_SN 736 bp
AAAGCAAGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTACAAAGCCAACGTGA
AGGCCATCTGGGGCAAAATCCTCCCTAAATCCGATGAGATTGGAGAACAGGCTCTTTCCAGGATGCTTGTTGTCTACCCC
CAGACCAAGGCCTACTTCTCCCACTGGGCTTCCGTTGCCCCCGGTTCCGCTCCAGTGAAGAAGCACGGCATCACCATCAT
GAATCAGATCGATGAATGTGTTGGCAACTTGGACGATCTCTTTGGTTTCTTGACCAAGCTCAGTGAACTGCACGCCACCA
AGCTGAGGGTGGACCCCACCAACTTCAAGATCCTGGCTCACAACCTGATTGTGGTCATTGCCGCCTACTTCCCTGCCGAA
TTCACCCCCGAGATCCACCTGTCCGTGGACAAGTTCCTGCAGCAACTGGCTCTGGCCCTGGCGGAGAAGTACCGCTAAAC
CGGAGTTCAGCTGCTAAACTGTCATCAGAGCTACAAGCTCTCACAGTCTTACATCAGAATTATACGTTCATCCATGTTTC
TCAAACGTAATGTCCTTCCGATATTGACACACTGTCAATAAACCTAAATTAAACCCAAAAAAAAAAAAAAAAAAAAAAAG
TACTCTGCGTTGATACCACTGCTTGCCCTATAGTGAGTCGTATTAGAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGA
GCATGCGACGTCGGGC

>HBaIV_RACE2_SN 756 bp
TATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTGTGAAGAAGCACGGCATCACCATCATGAATCAGATCGA
TGAATGTGTTGGCAACTTGGACGATCTCTTTGGTTTCTTGACCAAGCTCAGTGAACTGCACGCCACCAAGCTGAGGGTGG
ACCCCACCAACTTCAAGATCCTGGCTCACAACCTGATTGTGGTCATTGCCGCCTACTTCCCTGCCGAATTCACCCCCGAG
ATCCACCTGTCCGTGGACAAGTTCCTGCAGCAACTGGCTCTGGCCCTGGCGGAGAAGTACCGCTAAACCGGAGTTCAGCT
GCTAAACTGTCGTCAGAGCTACAAGCTCTCACAGTCTTACATCAGAATTAGACGTTCATCCATGTTTCTCAAACGTAATG
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AAAAAAAAAAAAAAAGGTACTCTGCGTTGATACCACTGCTTGCCCTATAGTGAGTCGTATTAAAATCGAATTCCCGCGGCC
GCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTT
TACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTTGCAGCACATCCCCCTTTTCGCCAGCTGGG
CGTAATAGCGAAAGAGGCCCGCACCGATCGCCCTTT

>HBB_RACE2_SN 745 bp
ATTATCAAGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTGTGGGGAAAGATCA
GCGTGGATGAGATCGGACCCCAGGCCCTGGCCAGACTTCTGATCGTGTCTCCATGGACTCAGAGACACTTCAGCACCTTC
GGCAACCTGTCCACACCCGCTGCCATCATGGGTAACCCCGCCGTGGCCAAGCACGGAAAGACCGTGATGCACGGACTGGA
CAGAGCTGTGCAGAACCTGGATGACATCAAGAACACCTATACTGCACTGAGTGTGATGCACTCCGAGAAACTGCACGTGG
ATCCCGACAACTTCAGGCTCCTCGCCGACTGCATCACCGTGTGCGTGGCCGCCAAGCTCGGTCCCGCCGTTTTCAGTGCT
GATACTCAGGAAGCCTTCCAGAAGTTCCTGGCTGTCGTTGTGTCCGCTCTTGGCAGACAGTACCACTAGAGCATCACTCG
ACAGCATCAATATGGAAGAGAGATGACACTCCAACTCCAGTCTGTTAGGCTGGAAGCTGTGCCATAGCTACACATATTGA
AAATAATAAAATACATGTAAAAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTTGCC
CTATAGTGAGTCGTATTAGAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCC
TATAGTGAGTCGTATTACAATTCAC

>HBB_RACE3_SN 762 bp
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GGGAAAGATCAGCGTGGATGAGATCGGACCCCAGGCCCTGGCCAGACTTCTGATCGTGTCTCCATGGACTCAGAGACACT
TCAGCACCTTCGGCAACCTGTCCACACCCGCTGCCATCATGGGTAACCCCGCCGTGGCCAAGCACGGAAAGACCGTGATG
CACGGACTGGACAGAGCTGTGCAGAACCTGGATGACATCAAGAACACCTATACTGCACTGAGTGTGATGCACTCCGAGAA
ACTGCACGTGGATCCCGACAACTTCAGGCTCCTTGCCGACTGCATCACCGTGTGCGTGGCGGCCAAGCTCGGTCCCACCG
CTTTCAGTGCTGATACTCAGGAAGCCTTCCAGAAGTTCCTGGCTGTCGTTGTGTCCGCTCTTGGCAGACAGTACCACTAG
AGCATCACTCGACAGCATCAATATGGAAGAGAGATGACACTCCAACTCCAGTCTGTTAGGCTGGAAGCTGTGCCATAGCT
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GTATTACAATTCACTGGGCCGTCGTTTTACAACGTCGTGACT