Thiaminase Activity of Crucian Carp * Carassius carassius Injected with a Bacterial Fish Pathogen, Aeromonas salmonicida subsp. salmonicida

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Abstract.—Dietary thiaminase I is a cause of thiamine deficiency in animals. The physiological significance of thiaminase in the organisms containing this enzyme is not known, nor are the factors causing variation in their thiaminase activity. Tests were performed to evaluate the effect a pathogen might have on thiaminase activity in fish, when analyzed both with a cosubstrate added (CATA tests) and no cosubstrate added (NCATA tests). Pyridine is known as a cosubstrate specific for thiaminase I activity that does not accelerate thiaminase II activity. Crucian carp * Carassius carassius known to harbor thiaminase I activity were injected intramuscularly with live Aeromonas salmonicida, a pathogenic bacterium of fish. For comparison, other groups were injected with formalin-killed bacteria and phosphate-buffered saline, respectively; an untreated group of fish was kept as a control. The bacteria did not contain any thiaminase activity. Significantly higher thiaminase activities (CATA and NCATA) were measured in all tissues (whole blood, injected muscle, uninjected muscle, and whole fish homogenates) of fish injected with live * Aeromonas salmonicida than in the saline-injected and the uninjected groups. The thiaminase activity of blood and that in the injected, inflamed muscle tissue followed different allocation patterns in fish injected with live * Aeromonas salmonicida. The amount of thiaminase I enzyme appeared to be elevated in the whole blood of injected fish in the absence of natural cosubstrate(s). The thiaminase activity of the injected, inflamed muscle suggested that both the amount of thiaminase enzyme and some yet-identified natural cosubstrate(s) were elevated. This suggests that in addition to the enzyme, some cosubstrate(s) of fish or pathogen origin play a regulatory role in the so-far-unknown physiological significance of thiaminase I activity in vivo. It is suggested that the health of fish should be considered when searching for factor(s) affecting its thiaminase activity.

Thiaminase I (enzyme number 2.5.1.2; IUBMB 1992) is a transferase-type enzyme that catalyzes the displacement of the thiazole moiety of thiamine (vitamin B_1) by a wide variety of nucleophiles: aromatic primary amines, heterocyclic compounds, and sulfhydryl compounds (i.e., co-substrates) (Fujita 1954). Thiaminase activity occurs among cyanobacteria (Arsan and Malyarevskaya 1969), plants, bacteria (Fujita 1954), invertebrates (Birger et al. 1973; Nishimune et al. 2000), shellfish, and fish (Melnick et al. 1945; Harris 1951; Fujita 1954; Greigh and Gaedinger 1971). The other thiaminase, thiaminase II (3.5.99.2), that catalyzes the hydrolysis of thiamine occurs exclusively in microorganisms (Wittliff and Airth 1970). While the thiaminase II in microorganisms seems to be involved in the thiamine salvage pathway (Onozuka et al. 2007), the physiological significance of thiaminase I in species that contain the enzyme is not known (Harris 1951; Fujita 1954; Evans 1975; Sato et al. 1994; Costello et al. 1996; Boś and Kozik 2000; Onozuka et al. 2007) and consequently there is a lack of knowledge concerning the possible agents or factors that cause the wide variation in thiaminase activity observed within organisms. The presence of thiaminase in the diet has been associated with the occurrence of thiamine deficiency symptoms among mammals, including humans (Green and Evans 1940; Evans 1975; Chick et al. 1989; Earl and McCleary 1994; Nishimune et al. 2000), and fish (Saunders and Henderson 1974; Fisher et al. 1996, 1998a, 1998b; Fitzsimons and Brown 1998; Honeyfield et al. 2005).

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217
Maternally transferred thiamine deficiency is the cause of the yolk sac fry mortality syndromes such as early mortality syndrome (EMS) and Cayuga syndrome (CS) reported in several species of salmonines in North America and of the M74 syndrome of Atlantic salmon *Salmo salar* in the Baltic Sea (Bylund and Lerche 1995; Fisher et al. 1996, 1998a; Amcoff et al. 1998; Brown et al. 1998; Koski et al. 1999). The immediate cause of the thiamine deficiency of salmonines in North America is a diet containing a nonnative, thiaminase-rich prey fish species, alewife *Alosa pseudoharengus* (Fitzsimons and Brown 1998; Honeyfield et al. 2005). Baltic herring *Clupea harengus membras* and sprat *Sprattus sprattus*, the major prey species of Atlantic salmon in the Baltic Sea (Karlsson et al. 1999), also contain thiaminase (Melnick et al. 1945; Him and Pekkanen 1975; Anglesea and Jackson 1985; Wistbacka et al. 2002). Unlike in North America, where nonnative prey species appear to be responsible for the thiamine deficiency, in the case of Baltic Sea salmon the native herring and sprat appear to be responsible, even though they have apparently coexisted with Baltic Sea salmon for several millennia. Nevertheless, the M74 syndrome in Baltic Sea salmon seems to be a recent phenomenon, suggesting qualitative changes in either herring, sprat, or both. Available data from salmon hatcheries indicate that no serious M74 outbreak occurred during the period 1928–1963 (Hansson et al. 2001).

Of the major prey species of Baltic salmon—herring, sprat, and threespine stickleback *Gasterosteus aculeatus*—the highest thiaminase activity has been measured in herring. Such activity varied widely even in specimens from single catches from the same areas (Wistbacka et al. 2002; Wistbacka and Bylund 2008). Similarly, wide variations in total thiaminase I activity have routinely been observed among forage fishes collected in single samplings at particular sites in North American lakes (Fitzsimons et al. 2005a; Tillitt et al. 2005; Zajicek et al. 2005). The causes of variation in thiaminase activity have not been identified, but a number of factors may be involved.

An increase in thiaminase I activity was experimentally induced in a plant, nardoo *Marsilea drummondii*, by cutting off old fronds (Chick et al. 1989). In experimental work by Malyarevskaya et al. (1970, 1972) and Malyarevskaya (1983), thiaminase activity increased in freshwater fish exposed to lethal concentrations of blue-green algae. Arsan and Malyarevskaya (1969) measured the highest thiaminase activity in silver carp *Hypophthalmichthys molitrix* fed the cyanobacterium *Microcystis aeruginosa* and less in those fed the green alga *Volvox aureus*, protococoid algae, daphnia *Daphnia magna*, and those given all these items mixed. The lowest enzyme activity was measured in fish that did not receive food.

The thiaminase activity in recently ingested (undigested) herring sampled from salmon stomachs was significantly higher than that in herring from net catches from the same area of the sea, suggesting that salmon are selectively feeding on herring with high thiaminase activity. We suggested that this was linked to the health status of the prey fish ingested (Wistbacka et al. 2002; Wistbacka and Bylund 2008). In a tank study the thiaminase activity in herring was significantly elevated even over a very short period, and the results indicated that herring with visible disease symptoms (darkened skin, abnormal swimming) had elevated thiaminase activity compared with herring with no visible symptoms (Wistbacka and Bylund 2008).

Based on the observations cited above, elevated thiaminase activity in an organism might be a response to adverse effects on the health of that organism. The objective of our study was to determine whether infection by a fish pathogen alters the thiaminase activity in a fish species known to contain thiaminase I, the crucian carp *Carassius carassius* (Fujita 1954). The crucian carp was chosen because it is more robust and easier to handle than the marine prey fish of Baltic Sea salmon and because it is a freshwater species that is more easily kept in the freshwater facilities we had available. Moreover, this carp was the first vertebrate species unequivocally shown to have thiaminase I activity that could be separated into a heat-labile, nondialyzable enzyme (“apoenzyme”) fraction and a heat-stable dialyzable cosubstrate (“coenzyme”) fraction (Kramitz and Woolley 1944). The distribution of thiaminase activity in different tissues of crucian carp is well described (Sealock et al. 1943), the highest thiaminase activities being found in the visceral tissues (spleen, liver, pancreas, and gastrointestinal tract) and gills. Thiaminase activity is almost absent from the muscles and swim bladder. Moreover, the carp is so far the only vertebrate species from which a homogeneous thiaminase I preparation has been purified (Boš and Kozik 2000).

**Methods**

**Fish and Pathogen**

Crucian carp were collected from a natural pond close to the city of Turku, Finland, and acclimated to laboratory conditions over an 8-week period before the trials. They were kept in flow-through groundwater tanks at 15.0°C and fed a commercial trout food. A pathogenic strain (P41-05) of *Aeromonas salmonicida* subsp. *salmonicida* (hereafter, *A. salmonicida*) isolated...
during an outbreak of clinical furunculosis in farmed whitefish *Coregonus lavaretus* (known as powan in North America) was used for pathogen exposure.

**Trial I.**—Colonies of *A. salmonicida* from tryptic soy agar (TSA; Difco, Sparks, Maryland) plates were incubated in 40 mL of tryptic soy broth (Difco) at 15°C for 48 h and then centrifuged; the remaining pellet was washed once with 40 mL of 0.9% phosphate-buffered saline (PBS; pH 7.2). The pellet was resuspended to a final volume of 8 mL, giving a bacterial density of $3 \times 10^8$ colony-forming units (CFU)/mL using McFarland standards (MacFaddin 1980). This suspension was divided into two equal volumes of 4 mL each and centrifuged. Each pellet was resuspended to 4 mL, one with PBS and the other with a 4% formalin solution, and incubated at 15°C for 1 h. After centrifugation, the pellets were resuspended and centrifuged twice with the PBS solution. The final suspensions were adjusted with PBS to the same optical density. The suspensions were further diluted in series by 10:1 with PBS; the final bacterial density was $1.9 \times 10^5$ CFU/mL. The volume injected in each fish was 0.1 mL ($1.9 \times 10^4$ CFU/fish).

A total of 60 specimens of crucian carp (mean weight, 18.4 g) were divided equally into 12 aquaria (5 fish/aquarium), each aquarium containing 40 L of aerated groundwater. Each treatment group comprised the fish in 3 aquaria, that is, 15 specimens. The fish were anesthetized with benzocaine (10 g/L) and added to the water, giving a final benzocaine concentration of 0.4%. One group was injected intramuscularly in the left lateral musculature with live *A. salmonicida* in PBS (LA), the second group with formalin-treated *A. salmonicida* in PBS (FA), and the third group with 0.1 mL of PBS; the fourth group was left as an anesthetized but uninjected control. The fish in all groups were sacrificed by a blow to the head 96 h posttreatment. Blood was immediately sampled from the caudal vein into an Eppendorf tube and stored frozen along with the fish in plastic bags at $-20^\circ$C until analyzed.

**Trial II.**—A total of 60 specimens of crucian carp were divided equally into 12 aquaria, each containing 40 L of aerated groundwater. The fish were divided into three treatment groups (20 fish per group) in 4 aquaria. The pathogen *A. salmonicida* was cultured and treated as in trial I. One group (LA) was injected in the left lateral musculature with the living pathogen ($1.6 \times 10^3$ CFU/fish) suspended in 0.1 mL of PBS and the second group with 0.1 mL of PBS only; the third group was anesthetized but kept as an uninjected control. One fish from each aquarium was sacrificed at 24, 48, 72, 96, and 168 h posttreatment. Sampling and sample storage were the same as in trial I.

**Sample Preparation**

*A. salmonicida.*—Samples for thiaminase analysis of the pathogen were prepared by collecting *A. salmonicida* colonies from TSA plates. Two parallel samples of 0.07 g were weighed in round-bottom glass tubes and homogenized in 2.0 mL of citrate phosphate buffer (0.1 M, pH 6.4) on ice. No further dilution of the pathogen samples was done.

Muscle and blood samples.—The epidermis and subcutaneous tissues were carefully removed from the fish. A piece of the fillet from the left side, including the visible injection site, and a similar piece from the un.injected right side were separated from the fish when the fish was still frozen. Of these muscle samples, 0.5 g was weighed out and homogenized on ice in 4.0 mL of citrate phosphate buffer in a round-bottom glass tube with a pestle homogenizer. Of the blood samples, 0.1 g was weighed out and homogenized in 4.0 mL of citrate phosphate buffer. No further dilution of the blood and muscle samples was done.

Whole fish homogenate.—The whole fish were cut into approximately 0.5-cm-thin slices while still frozen and homogenized with a blade homogenizer; the temperature of the sample did not exceed $0^\circ$C. The samples were refrozen and stored at $-20^\circ$C until analyzed. For analysis, 2.0 g of the fish homogenate was weighed out and transferred to a round-bottom glass tube, then further homogenized on ice with a pestle homogenizer in 4.0 mL of citrate phosphate buffer and centrifuged for 20 min at 3,000 × gravity at 4°C. A volume of 0.3 mL of the supernatant was further diluted with 1.8 mL of citrate phosphate buffer.

Reisolation of pathogens.—Reisolation was attempted from the visible injection site of the left muscle and the same site in uninjected fish (trial I: 40 fish; trial II: 30 fish) in parallel with preparation of the samples for the thiaminase assay (as the sample was still frozen). The formation of colonies producing brown pigment on TSA plates was considered positive for *A. salmonicida*.

Thiaminase assay.—Thiaminase activity was analyzed using the radioactive thiamine method of Edwin and Jackman (1974), with minor modifications. As thiamine is destroyed by thiamine I only in the presence of a cosubstrate (i.e., a substance that is exchangeable with the thiazole moiety of thiamine), thiaminase activity was determined both with an artificial cosubstrate, pyridine, added (CATA) or no cosubstrate added (NCATA). In addition to thiaminase I and II, it is known that sulfite has thiamine-destroying properties (Leichter and Joslyn 1969; Jaroensanti and Panijpan 1981). Furthermore, it has been suggested that
a heat-stable thiamine-destroying factor occurs in common carp *Cyprinus carpio* and several other species of fish (Somogyi and Kundig 1963; Hilker and Peter 1966). However, this factor was later shown to be a thiamine-modifying factor that interferes with the method used for the analysis of thiamine, not a true thiamine-destroying factor (Hilker 1976).

The ambient or actual thiaminase activity (NCATA tests) reflects the rate of thiamine destruction at the time the sample was analyzed. Thus, the reaction rate was dependant on the amounts of the enzyme and naturally occurring thiaminase I–suitable cosubstrates (and/or thiaminase II–like or other thiamine-splitting compounds such as sulfite). For the CATA tests, the cosubstrate pyridine was added to the sample. Pyridine does not accelerate the activity of thiaminase II (Fujita 1954), but it is one of the most effective cosubstrates among those Bos and Kozik (2000) tested for purified carp thiaminase I. Pyridine also has no accelerating effect on the thiaminolytic activity of sulfite (S. Wistbacka, unpublished data). Accordingly, the CATA tests provided a measure of the thiaminase activity if the concentration of naturally occurring cosubstrates was not a limiting factor (Thomas and Griffiths 1987). The thiaminase activity that is accelerated by the addition of pyridine is thus considered a specific measurement of thiaminase I activity.

Thiazole-2-¹⁴C–labeled thiamine (Amersham Pharmacia Biotech, Amersham, UK) with a specific activity of 25 mCi/mmol of thiamine and a total activity of 0.63 mCi/mL was dissolved in 0.1 N HCl to give a solution of 0.063 mCi/mL as a stock solution. Working solution I, for the NCATA analysis, was prepared by mixing 0.1 mL of stock solution with 100 mL of citrate phosphate buffer (0.1 M, pH 6.4) containing nonradioactive thiamine at a concentration of 14.8 nmol/mL. Working solution II, for the CATA analysis, was the same as working solution I but with 50 mM of pyridine added as a cosubstrate. The specific activity of the working solutions was 8,072 dpm (disintegration per minute) nmol of thiamine, and the thiamine concentration was 17.3 nmol/mL.

Volumes (0.5 mL) of the sample solutions were transferred into each of two centrifuge tubes and mixed with 0.5 mL of citrate phosphate buffer instead of the sample in every batch analyzed. Ethyl acetate (4 mL) was added to each tube, the tubes were shaken vigorously to induce the absorption of the thiazole by the ethyl acetate, and then the tubes were centrifuged for 10 min at 3,000 gravity at 4°C. A 0.5-mL sample of the supernatant from each tube was transferred to a scintillation vial together with 5 mL of scintillation fluid (Ultra Gold F; Perkin Elmer, Boston, Massachusetts). The samples were analyzed with a Wallac 1410 liquid scintillation counter (Wallac Oy, Turku, Finland).

**Statistical analysis.**—The differences between treatments were analyzed for each sampled tissue with one-way analysis of variance (ANOVA) and Sheffé’s post hoc test. The differences between CATA and NCATA specimens within samples were analyzed with pairwise *t*-tests. All statistical analyses were carried out in Statistical Product and Service Solutions 11.5 for Windows.
TABLE 1.—Extended.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WB Activity</th>
<th>WFH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Activity</td>
<td>13.633 z (8.536)</td>
<td>28.908 z (4.472)</td>
</tr>
<tr>
<td>FA</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Activity</td>
<td>2.095 y (1.225)</td>
<td>12.339 y (1.645)</td>
</tr>
<tr>
<td>PBS</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Activity</td>
<td>1.813 z (1.902)</td>
<td>12.183 y (7.210)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Activity</td>
<td>0.842 y (0.531)</td>
<td>8.261 y (0.950)</td>
</tr>
</tbody>
</table>

Results

Thiaminase Activity of A. salmonicida

There was no detectable thiaminase activity in the fish pathogen used. The NCATA and CATA analyses for results that were slightly negative or slightly positive (~0.15 ± 0.09 [mean ± SD]) and 0.07 ± 0.11 nmol · g⁻¹ · min⁻¹, respectively).

Disease Signs in Fish and Reisolati on of the Pathogen

In trial I, signs of furunculosis were observed in fish injected with live A. salmonicida within 48 h posttreatment when one moribund fish was sacrificed. Signs of ulceration at the injection site were observed as raised scales and hemorrhages in the skin. After 72 h two additional moribund specimens from the same group were sacrificed. The formation of ulcers was observed in all fish injected with live A. salmonicida. No signs were observed among fish in the other groups. Reisolation of the pathogen was positive for 8 of the 10 fish examined in the group treated with live A. salmonicida and negative in all fish examined from the other categories. No visible differences were seen between fish treated with formalin-killed pathogens and those treated with physiological saline (PBS), but on necropsy the reddish injection channel was visible in fish in both groups.

In trial II, the batch of live bacteria injected into the fish muscle was reduced ten-fold in order to avoid fish mortality during the trial. Consequently, there were no signs of disease in any fish in this trial. There was no ulcer formation at the injection site of any fish, and reisolation of the pathogen was negative for all 30 fish examined, 10 of which were from the group injected with live A. salmonicida.

Thiaminase Activity of Crucian Carp in Trial I

The amounts of thiaminase activity in injected muscle (IM), un.injected muscle (UIM), whole blood (WB), and whole-fish homogenates (WHF) are presented in Table 1. The thiaminase activity (NCATA and CATA) of the LA group was significantly higher in all tissues sampled, as was that in the whole-fish homogenate. Slightly elevated thiaminase activity was also indicated in fish injected with formalin-killed A. salmonicida and fish injected with PBS relative to the control group. The difference was significant when comparing injected muscle samples from fish injected with formalin-killed A. salmonicida with muscle samples of untreated control fish. There was no significant difference between fish injected with formalin-killed bacteria and saline-injected fish.

Because pyridine accelerates thiaminase I activity but not thiaminase II activity, a significant difference between NCATA and CATA samples is a specific indication that thiaminase I is present in the sample. Thiaminase activity measured with no cosubstrate added reflects the level of the enzyme and natural thiaminase I cosubstrates present (and/or possible thiaminase II–like activity in the sample). There was a significant difference between NCATA and CATA fish in all samples and treatment groups in trial I (t-test; P < 0.05), except for the IM samples of fish treated with live A. salmonicida, where NCATA and CATA activities were at the same elevated level (13.02 and 13.04 nmol · g⁻¹ · min⁻¹, respectively).

The CATA activity in the blood samples of fish injected with live A. salmonicida were at the same level (13.63 nmol · g⁻¹ · min⁻¹) as the NCATA and CATA activities of the injected muscle, but the NCATA activity was considerably lower (0.583 nmol · g⁻¹ · min⁻¹), suggesting the absence or only limited quantities of thiaminase I–suitable cosubstrates in the blood. In fish injected with live A. salmonicida, the NCATA activity was significantly higher in the injected muscle than in whole-fish homogenates (pairwise t-test; df = 9, P < 0.001). No significant difference was found between the NCATA activity of injected muscle and that of whole-fish homogenates in the other treatment groups (P > 0.05).

Thiaminase Activity of Crucian Carp in Trial II

The aim of trial II was to determine the response with respect to thiaminase activity in fish over time. In each treatment group four specimens were sampled at 24, 48, 72, 96, and 168 h posttreatment. Two specimens were used for analysis of whole-fish homogenates and two for analysis of muscle samples, which consequently reduced the sample size. The
elevated CATA activity of muscle tissue injected with live *A. salmonicida* suggests that the proliferation of thiaminase activity is initiated within 24 h, but owing to the small sample size the difference from the PBS-injected and the control fish was significant only from 48 to 96 h (Table 2). The CATA activity was still elevated at 168 h but the difference was not significant.

Over time, the CATA values for the whole-fish homogenates resembles those for the injected muscle tissues (Table 3), and as in trial I, were higher in the whole-fish homogenate than in the muscle samples from fish in the same treatment group. The NCATA activity of the IM samples was elevated in the LA group compared with the control group, but the difference was significant only at 96 h (Table 2). However, as in trial I, the NCATA activity of the IM samples of the LA group did not reach the level of CATA activity, and the difference between NCATA and CATA activity in the muscle samples of all treatment groups was significant (paired *t*-test; df = 9, *P* < 0.01). As in trial I, the NCATA activity of the IM samples of the LA group was significantly higher than that of the WFH samples from the same treatment group (df = 9, *P* < 0.01). In contrast, in the untreated control fish, the NCATA activity of whole-fish homogenates was significantly higher than in the muscle (df = 9, *P* < 0.01). This suggests that thiaminase I–suitable cosubstrates (and/or thiaminase II–like activity) was concentrated at or allocated to the injection site in fish injected with the reduced amount of live *A. salmonicida*, albeit not enough to produce the level of CATA activity in trial I.

**Discussion**

It is now recognized that maternally transferred thiamine deficiency is the cause of yolk sac fry mortality in salmonines in North America (EMS and CS) as well as in salmon in the Baltic Sea (M74). The immediate cause of the thiamine deficiency in salmonine broodfish is the ingestion of thiaminase-rich prey fish—nonnative alewives in North America and native clupeids in the Baltic Sea. Thiamine deficiency has experimentally been induced in salmonines fed these prey fish species (Saunders and Henderson 1974; Honeyfield et al. 2005).

As stated in the introduction to this paper, elevated thiaminase activity may be related to weakened health among fish. The results obtained in the present study support this hypothesis, as experimental exposure of crucian carp to the pathogen *A. salmonicida* increased thiaminase activity in fish with and without clinical signs of furunculosis.

The physiological significance of thiaminase I is not known. It is so far unclear whether the enzyme

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**Table 2.**—Mean (SD) thiaminase activity (nmol·g⁻¹·min⁻¹) of injected muscle sampled at five times posttreatments (trial II). Significant differences between treatments are indicated by different letters (ANOVA and Sheffe’s post hoc test, *P* < 0.05), *N* = 2 throughout. See Table 1 for additional details.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>7.580 z (3.041)</td>
<td>11.895 z (1.949)</td>
<td>12.449 z (1.041)</td>
<td>12.515 z (0.489)</td>
<td>6.732 z (8.201)</td>
</tr>
<tr>
<td>PBS</td>
<td>1.220 z (0.706)</td>
<td>2.995 y (1.192)</td>
<td>0.885 y (0.341)</td>
<td>2.024 y (2.318)</td>
<td>0.729 z (0.901)</td>
</tr>
<tr>
<td>Control</td>
<td>0.153 z (0.052)</td>
<td>0.189 y (0.066)</td>
<td>0.169 y (0.029)</td>
<td>0.216 y (0.170)</td>
<td>0.109 z (0.052)</td>
</tr>
<tr>
<td>NCATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>1.080 z (0.841)</td>
<td>3.478 z (1.532)</td>
<td>6.719 z (5.834)</td>
<td>4.656 z (1.546)</td>
<td>3.066 z (4.157)</td>
</tr>
<tr>
<td>PBS</td>
<td>0.132 z (0.039)</td>
<td>0.277 z (0.161)</td>
<td>0.095 z (0.002)</td>
<td>0.280 y (0.283)</td>
<td>0.123 z (0.053)</td>
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<tr>
<td>Control</td>
<td>0.043 z (0.006)</td>
<td>0.048 z (0.001)</td>
<td>0.016 z (0.050)</td>
<td>0.042 y (0.057)</td>
<td>0.022 z (0.044)</td>
</tr>
</tbody>
</table>

**Table 3.**—Mean (SD) thiaminase activity (nmol·g⁻¹·min⁻¹) of whole-fish homogenates sampled at five times posttreatment (trial II). Significant differences between treatment groups are indicated by different letters (ANOVA and Sheffe’s post hoc test, *P* < 0.05); *N* = 2 throughout. See Table 1 for additional details.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
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</tr>
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<tr>
<td>CATA</td>
<td></td>
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<tr>
<td>LA</td>
<td>11.132 z (2.018)</td>
<td>17.823 z (0.388)</td>
<td>16.475 z (0.897)</td>
<td>20.414 z (7.492)</td>
<td>13.937 z (0.894)</td>
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<tr>
<td>PBS</td>
<td>5.982 z (0.885)</td>
<td>6.236 y (0.710)</td>
<td>5.021 y (0.264)</td>
<td>4.984 z (1.347)</td>
<td>4.901 y (0.204)</td>
</tr>
<tr>
<td>Control</td>
<td>6.653 z (2.064)</td>
<td>6.889 y (0.428)</td>
<td>4.718 y (0.666)</td>
<td>7.245 z (0.025)</td>
<td>5.935 y (1.065)</td>
</tr>
<tr>
<td>NCATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>0.392 z (0.206)</td>
<td>0.723 z (0.511)</td>
<td>0.234 z (0.119)</td>
<td>0.818 z (0.011)</td>
<td>0.279 z (0.147)</td>
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<tr>
<td>PBS</td>
<td>0.375 z (0.102)</td>
<td>0.143 z (0.113)</td>
<td>0.070 z (0.144)</td>
<td>0.167 x (0.022)</td>
<td>0.176 z (0.040)</td>
</tr>
<tr>
<td>Control</td>
<td>0.111 z (0.166)</td>
<td>0.192 z (0.047)</td>
<td>0.136 z (0.008)</td>
<td>0.234 y (0.003)</td>
<td>0.087 z (0.002)</td>
</tr>
</tbody>
</table>
demonstrated in fish is a native enzyme, is derived from external sources, or both. Honeyfield et al. (2002) isolated thiaminase-producing bacteria, mainly *Paenibacillus thiaminolyticus* (formerly Bacillus thiaminolyticus) and closely related bacteria, from the viscera of alewives, suggesting a nonnative source of thiaminase I in this fish species. Some of the enzymatic properties (e.g., temperature dependence) of the bacterial-derived thiaminase I protein were similar to those observed in crude extracts of alewives (Zajicek et al. 2005). On the other hand, Boš and Kozik (2000) found that the thiaminase I protein derived from carp liver had an unique N-terminal sequence that did not resemble any fragment of the enzyme from *P. thiaminolyticus*, although the enzymatic properties of the carp liver enzyme are very similar to those of the bacterial protein.

The pathogen injected into the fish tissue in our study did not have any thiaminase activity. It is therefore reasonable to suppose that the increased thiaminase activity in our fish was due to the expression and proliferation of a native enzyme in the fish. We cannot absolutely exclude the possibility of an accidental secondary invasion of, for example, thiaminase-producing microorganisms in the test fish, and therefore this statement is tentative. The enzyme level (CATA tests) in fish injected with the pathogen was elevated not only at the injection site but also in all of the other tissues analyzed.

The thiaminase activity measured with no cosubstrate added was barely detectable in whole blood, in marked contrast to that with the cosubstrate pyridine added. Since pyridine is a specific cosubstrate for thiaminase I, this is considered evidence of elevated thiaminase I in the blood of the pathogen-treated fish and consequently the relative absence of thiaminase I–suitable natural cosubstrates. In fish injected with the pathogen in trial I, the enzymatic reaction rates in samples of inflammatory muscle tissue were at the same high level with and without cosubstrate added (Table 1). The elevated level of thiaminase activity without the addition of pyridine suggests a high level of natural cosubstrate(s) in the inflamed muscle. Since we did not check for thiaminase II–like activity by measuring the remaining thiaminase activity after separation of the enzyme and cosubstrates by dialysis or precipitation (Krampitz and Woolley 1944; Fujita 1954), we are not able to exclude the possibility of such activity as a cause of the elevated NCATA values in injected muscle. Since so far thiaminase II has been found only among some microorganisms, elevated thiaminase II activity could theoretically stem from a secondary invasion of thiaminase II–producing microorganisms at the injection site. This is, however, speculation in the absence of positive evidence. Our results suggest that enough naturally occurring cosubstrate(s) for thiaminase I were present, particularly in the inflammatory tissue around the injection site. When fewer bacteria were injected (trial II), no ulcer formation or symptoms of furunculosis occurred, though the CATA activity at 96 h achieved the level of that in trial I while the NCATA activity was significantly lower than the CATA activity. These results suggest that there was some effect on NCATA activity associated with the bacterial dose injected, the proliferation of the pathogen, or cell damage by ulcers at the inflammatory site.

Further, in samples from uninfected muscle, whole blood, and whole-fish homogenate, NCATA activity was significantly lower than CATA activity, suggesting an insufficiency of a suitable natural cosubstrate. Although a cosubstrate was present in the inflammatory tissue around the site injected with live pathogens in trial I and at the injection site when no clear inflammation was visible in trial II, from the present results we are not able to state whether the cosubstrate is of host or pathogen origin. However, since thiaminase I requires a cosubstrate for its reaction, these results suggest that some yet unidentified cosubstrate(s) may play a significant role in vivo thiaminase I activity in fish.

Sato et al. (1994) found that the thiaminase I in the kidney and spleen of common carp was localized in the lysosomes. Lysosomes are dynamic organelles that receive and degrade macromolecules from the secretory, endocytic, autophagic, and phagocytic membrane-trafficking pathways (Luzio et al. 2007). The innate or unspecific immune defense of fish against bacteria includes a broad spectrum of anti-microbial substances and acute-phase proteins, nonclassical complement activation, release of cytokines, inflammation, and phagocytosis, including the formation of lysosomes (Ellis 2001; Magnadóttir 2004).

The response in terms of increased thiaminase activity in crucian carp over time that we found (Tables 2, 3) is consistent with the time course of early-defense responses observed in rainbow trout *Oncorhyncus mykiss* after intraperitoneal injection with bacteria (Ellis 2001 and references therein). Even if we cannot definitively state that the assumed native thiaminase I participates in the immune defense of fish, the suggestion is worth serious consideration when examining the physiological role of thiaminase I. In this context the findings of Pirie (1959) make sense; that author found that the infective nucleic acid of tobacco mosaic virus lost its infectivity when treated with a thiaminase preparation in the presence of thiamine but not with either alone.
Further, pyridine is a constituent of many natural compounds, that are known for their antimicrobial properties, the simplest with structural similarities to the pyrimidine moiety of thiamine (Lagoja 2005). In addition to the antibiotic mechanisms of their antimicrobial activity, macrophages are able to deprive intracellular pathogens of required nutrients (Appelberg 2006), and this may be a role that thiaminase I plays in connection with the lysosomes. In addition, if the targets of thiaminase in the inflamed tissue are the damaged cells of the host, thiaminase may deprive the cell of thiamine to induce apoptosis (Fulton and Lai 2003).

The inflammatory reaction following intramuscular injection seems to be associated with an increased thiaminase activity. The thiaminase activity (CATA tests) of crucian carp injected with formalin-killed bacteria or PBS were slightly elevated over those of untreated fish (Table 1). There was, however, no significant difference in thiaminase activity between fish injected with formalin-killed bacteria and those injected with PBS. In a recent study on herring, thiaminase activity with pyridine added as cosubstrate was significantly greater in the erythrocytes and muscle of fish injected with a water-based vaccine containing inactivated Vibrio anguillarum than in untreated control fish. Negative correlations were observed between erythrocyte and muscle thiaminase activity (CATA tests) and liver CYP1A mRNA expression, supporting a link between thiaminase and detoxification in herring (R. Waagbø, S. Wistbacka and P. A. Olsvik, National Institute of Nutrition and Seafood Research, Bergen, Norway, unpublished data). Albeit their study did not include injection with live bacteria or saline, their results are otherwise consistent with the results of this study, suggesting that thiaminase activity responds similarly to injection in both herring and crucian carp. Despite our insufficient knowledge of the origin of and significance of thiaminase I in hosts possessing this enzyme, we conclude that fish pathogens are potential agents for triggering thiaminase activity in fish.

In addition to low thiamine levels, M74 is associated with reduced levels of nonenzymatic antioxidants (AOX), such as carotenoids, tocopherol, and ubiquinone (Börjesson and Norrgren 1997; Lundström et al. 1999a, 1999b; Pettersson and Lignell 1999b). Both the adult salmon on feeding migrations in the Baltic Sea and their progeny show signs characteristic of oxidative stress (Vuori 2007). As suggested by our results, increased thiaminase activity in fish might be related to activation of their immune system. A well-known component of the innate immune system is the respiratory burst reaction, when reactive oxygen species (ROS) are produced. The inflammatory response in critically ill human patients is associated with increased lipid peroxidation and reduced carotenoid concentrations (Oldham and Bowen 1998; Quasim et al. 2003). It therefore seems reasonable that unhealthy prey (with an activated immune response) would be a significant source of thiaminase and might well influence the AOX–ROS balance in the diet of Baltic salmon and thus that of the fish themselves.

Since 1974, when the fry mortality syndrome was first observed, a variation in sprat biomass in the Baltic Sea has been found to correlate significantly with the prevalence of M74 (Karlsson et al. 1999). The factors and trophic cascades that are behind the rapid increase in sprat biomass in the Baltic Sea since the 1980s are well described (Hansson and Rudstam 1990; Thunow 1997; Hansson et al. 2007; Ósterblom et al. 2007; Casini et al. 2008). High stock density among prey brought on, for example, by overfishing of their predators, increases the risk of epidemics (Jackson et al. 2001; Lafferty 2004). In addition, the risk factors for an epidemic include poor body condition (Marty et al. 2003). The growth, condition, and lipid content of clupeids in the Baltic Sea seem to be associated with their abundance (Cardinale and Arrhenius 2000; Rönkkönen et al. 2004; Casini et al. 2006), and the prevalence of M74 is associated with these factors (Wistbacka and Bylund 2008). The condition factor of Baltic herring and sprat as well as the lipid content of Baltic herring reached a minimum in the mid-1990s (Bignert et al. 2006; Casini et al. 2006), that is, when the prevalence of M74 reached its maximum (Bengtsson et al. 1999; Ikonen 2006). Fitzsimons et al. (2005a) reported a significant negative correlation between the mean thiaminase activity and lipid content of alewives from various water bodies. However, though feeding alewives diets of differing lipid contents resulting in different lipid contents in the fish, there was no significant relationship between thiaminase activity and either dietary or tissue lipid level. It was thought that the correlation observed earlier might represent auto-correlation with some as yet unidentified factor or factors (Fitzsimons et al. 2005b).

As suggested by our results, thiaminase activity may increase in fish infected with pathogens whether disease symptoms are present or not. So far it is not possible to associate any pathogens recorded from salmon prey fish with their thiaminase activity, biomass, or the prevalence of M74 owing to the lack of continuous surveillance both of diseases and of thiaminase activity among clupeids in the Baltic Sea. Investigations of diseases and parasites in clupeids in this region have focused on infestations with the parasitic nematode Anisakis sp., which appears to be
largely restricted to the southwestern parts of the Baltic Sea, and to the spring-spawning herring population with feeding areas in Kattegat and Skagerrak straits and the North Sea. Infection with the flagellate protozoan *Ichtyophonus* sp. was noted for the first time in Baltic herring in summer 1991. The infested herring belonged to the same western spring-spawning stock characterized by a high prevalence of *Anisakis* larvae (HÉL-COM 1996). Lymphocystis disease was demonstrated in herring from the Swedish coast opposite the main basin of the Baltic Sea and the Gulf of Bothnia in a 1971–1972 study by Aneer and Ljungberg (1976). The prevalence of the disease varied from 0 to 2.7% in different samples. In 1994 Grygiel (1999) recorded an increasing prevalence of lymphocystis in herring samples along an eastward transect from the southwestern Baltic Sea to the Gulf of Finland; the prevalence ranged from 0 to 7.4%, although the disease was absent from sprat. In general, he found higher prevalences of disease and pathological emaciation in the western Baltic Sea to the Gulf of Finland; the prevalence ranged from 0 to 7.4%, although the disease was absent from sprat. In general, he found higher prevalences of disease and pathological emaciation in herring than in sprat. Lönnström et al. (1994) isolated *Pseudomonas anguilliseptica* from Baltic herring with eye lesions caught in the Gulf of Bothnia in 1993, but no prevalence of eye lesions among the herring was reported.

In addition to focusing on synoptic studies of the disease symptoms of clupeids in the Baltic Sea, researchers have been concerned that viral hemorrhagic septicemia virus (VHSV) could be transferred from wild to farmed fish (Mortensen et al. 1999). This concern resulted in efforts to isolate VHSV from wild fish. Mortensen et al. (1999) isolated VHSV from several species of wild marine fish, including herring and sprat, in the southern and southwestern Baltic Sea in 1996–1997. In a follow-up study in 1998–2002, Skall et al. (2005a, b) calculated VHSV prevalences between 5.6% and 7.8% in sprat and 0.0% and 16.7% in herring in waters surrounding Bornholm Island in the southwestern Baltic Sea. In an ongoing project, the Finnish food safety authority (EVIRA) has so far isolated VHSV in 48 of 180 pools of herring (10 fish/pool) collected in spring 2004 from the Archipelago Sea in southwestern Finland (L. Sihvonen, EVIRA, personal communication), suggesting a mean prevalence of about 25%. Most of the VHSV isolations from the southern Baltic Sea came from fish without clinical signs of viral hemorrhagic septicemia; it is not known whether this is because the Baltic strain of VHSV does not cause disease in wild clupeid fish or VHSV-positive fish are carriers that have survived VHSV infection (Skall et al. 2005a). The North American strain of VHSV is highly pathogenic to Pacific herring *Clupea pallasi* (Kocan et al. 1997) and has been a significant factor in regulating the Pacific herring stock (Meyers et al. 1999; Marty et al. 2003). Confinement of wild-captured and seemingly healthy Pacific herring in net pens and laboratory tanks has resulted in the increased prevalence and tissue titers of VHSV within a few days (Hersherberger et al. 1999, 2006). Interestingly, we observed a rapid increase in thiaminase activity among Baltic herring confined in a fish tank in spring 2003 (Wistbacka and Bylund 2008), that is, in wild fish captured from the Archipelago Sea, where high prevalences of VHSV were recorded the following spring. Despite the observed morbidity among the confined fish in the tank experiment, we did not continue with further pathological examination of the fish.

The results from this work and previous studies (Wistbacka et al. 2002; Wistbacka and Bylund 2008) suggest that in looking for the so-far-unidentified factor(s) influencing thiaminase activity in the prey fish of Baltic salmon, we should focus more on the state of health of these fish.

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