THE OCCURRENCE AND PREVENTION OF THE M74 SYNDROME, A THIAMINE DEFICIENCY DISEASE IN BALTIC SALMON

Perttu Koski

ACADEMIC DISSERTATION
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The occurrence and prevention of the M74 syndrome, a thiamine deficiency disease in Baltic salmon

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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>α-KGDH</td>
<td>α-ketoglutaratedehydrogenase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variance = SD/mean</td>
</tr>
<tr>
<td>EMS</td>
<td>Early mortality syndrome</td>
</tr>
<tr>
<td>EROD</td>
<td>Ethoxyresorufin-O-deethylase</td>
</tr>
<tr>
<td>GSI</td>
<td>Gonadosomatic index</td>
</tr>
<tr>
<td>HSI</td>
<td>Hepatosomatic index</td>
</tr>
<tr>
<td>MSW</td>
<td>Multi-sea-winter</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TH</td>
<td>Free thiamine</td>
</tr>
<tr>
<td>TH-HCl</td>
<td>Thiamine hydrochloride</td>
</tr>
<tr>
<td>TMP</td>
<td>Thiamine monophosphate</td>
</tr>
<tr>
<td>TPP</td>
<td>Thiamine pyrophosphate = thiamine diphosphate</td>
</tr>
<tr>
<td>TTH</td>
<td>Total thiamine</td>
</tr>
<tr>
<td>TTP</td>
<td>Thiamine triphosphate</td>
</tr>
<tr>
<td>( V_d )</td>
<td>Volume of distribution</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, referred to in the text by Roman numerals I-IV.


1. ABSTRACT

The M74 syndrome is a serious disease affecting feral and wild stocks of Baltic salmon, and can result in high mortality rates in yolk-sac fry. A linkage between the syndrome and a deficiency of thiamine has been proposed. This work aimed at describing the occurrence of the syndrome in important Bothnian Bay rivers, examining the distribution of thiamine in Baltic salmon and exploring the possible prevention of the syndrome using thiamine supplements. M74 syndrome was shown to be prevalent in the feral and wild Baltic salmon stocks of the Bothnian Bay rivers of Finland in the years 1994-96. It was not found to exist in the River Teno Atlantic salmon or the stock of the Latvian River Daugava. Fairly precise concentrations of total thiamine in the newly stripped eggs (threshold value ca. 0.35 µg g⁻¹) were shown to determine whether the yolk-sac fry developed M74 syndrome. The thiamine concentrations of the liver and white muscle tissues of the female fish were shown to correlate with the concentrations in the eggs of the female broodfish. Concentrations in the white muscle tissue were better correlated than those in the liver with the concentrations in the eggs, especially in the low concentration range, which is typical for fish having offspring with M74 syndrome. The white muscle tissue was shown to be a potential store for thiamine in the salmon broodfish during the spawning migration, when the fish does not obtain thiamine via food. A minor male effect was shown to exist in addition to the known major female effect on the development of M74 syndrome in their offspring.

The study of the pharmacokinetics of thiamine in the Baltic salmon broodfish revealed considerable differences between farmed and feral female broodfish. The total thiamine concentrations in the eggs and white muscle of feral females were approximately half of those in the farmed fish after intraperitoneal injection of thiamine hydrochloride. The relative inefficiency of the incorporation of thiamine into the eggs in the feral fish probably contributes to the development of M74 syndrome. The concentrations after intraperitoneal injection were, however, high in both feral and farmed females, which may indicate active transport into the cells or thiamine binding in these tissues.

The prevention of M74 syndrome in yolk-sac fry by bathing them in thiamine hydrochloride was shown to be dose-dependent. This and the corresponding effect on the thiamine concentrations in the eggs of the feral broodfish after thiamine hydrochloride injection emphasize the role of the thiamine supply of the female salmon in the development of M74 syndrome. An interaction between astaxanthine and thiamine may occur in the developing embryo or yolk-sac fry. The thiamine treatments were regarded as safe for the yolk-sac fry and broodfish. An intraperitoneal dose of 20 mg thiamine hydrochloride per kg broodfish was shown to be sufficient to elevate the concentrations in the eggs above the threshold value of M74 syndrome in their offspring. This is ca. one fifth of the dose used until now. Injections of caught and released wild salmon broodfish were performed during the studies. However, the effect on the smolt production in rivers where salmon suffer M74 syndrome needs to be further investigated.

Key words: Thiamine, astaxanthine, Baltic salmon, Salmo salar, M74 syndrome, pharmacokinetics, prevention
The occurrence and prevention of the M74 syndrome, a thiamine deficiency disease in Baltic salmon

2. INTRODUCTION AND REVIEW OF THE LITERATURE

2.1. Thiamine deficiency diseases of salmonid fish

2.1.1. Mortality syndromes

The first descriptions of thiamine deficiency disease in salmonid fish originate from the 1940s-60s, when farmed fish were fed with moist diets containing enzymes, thiaminases, that destroyed the thiamine in the feed (Christensen, 1966; Halver, 1989). These descriptions dealt with thiamine deficiency in growing fish. Thiamine deficiency has been almost unknown in food fish farming for decades, since present day feeds incorporate a vitamin premix containing recommended levels (National Research Council 1993; Halver, 1989) of thiamine.

Norrgren et al. (1993) described mortality among yolk-sac fry of Baltic salmon (the Baltic group of *Salmo salar* L.), which was termed M74. It had been observed in Sweden since 1974 and in Finland since 1992 (Koski et al. 1996; Soivio 1996). The name M74 comes from the Swedish word "miljöbetingad", meaning environmentally caused, and the first year when the disease was recognized (Norrgren et al., 1993). The syndrome has been observed in the fry of wild and feral Baltic salmon in several rivers of Sweden and Finland, but not in the salmon of Latvian or Polish Baltic salmon rivers (Mitans, 1994; Bartel, 1996). In addition to Baltic salmon, M74 has also been reported to occur sporadically in sea trout (*Salmo trutta* m. *trutta* (L.)) in the Baltic Sea (Amcoff et al., 1999a; Landergren et al., 1999). The locations and species of wild and feral salmonid fish fry in which thiamine deficiency diseases have been reported are presented in Figure 1.

Early Mortality Syndrome (EMS) was present in the offspring of the wild salmonids of Lake Michigan in the 1960s (Marcquenski and Brown, 1997). It has since been reported to occur in four of the Great Lakes of Canada and the United States. The salmonid species affected include Coho salmon (*Oncorhynchus kisutch* (Walbaum)), Chinook salmon (*O. tshawytscha* (Walbaum)), steelhead or rainbow trout (*Oncorhynchus mykiss* Robertson), Atlantic salmon, brown trout (*Salmo trutta* m. *lacustris* (L.)) and lake trout (*Salvelinus namaycush* (Walbaum)) (Skea et al., 1985; Fisher et al., 1996; Marcquenski and Brown, 1997; Hornung et al., 1998; Honeyfield et al., 1998; Johnson et al., 2002).

Land-locked Atlantic salmon of certain lakes in the State of New York, US, were described to suffer from almost total mortality of the first feeding fry Fisher et al. (1996 and 1998). This disease had been locally named Cayuga Syndrome according to one of the Finger Lakes, where the condition had been first observed.
Figure 1. Mortality syndromes related to thiamine deficiency reported in fry of different species of feral salmonids originating in the Great Lakes area of North America (left) and the Baltic Sea (right). Data based on the review of Marcquenski and Brown (1997) and 1: Norrgren et al. (1993), 2: Fisher et al. (1996), 3: Fisher et al. (1998), 4: Amcoff et al. (1999a), 5: Landergren et al. (1999), 6: Johnson et al. (2002).

All the above-mentioned disease syndromes were of unknown aetiology for at least some years before the role of thiamine deficiency in the disease(s) was established. In addition to the disease affecting yolk-sac fry, adult wild or feral salmonids have also been reported to die due to a thiamine deficiency: Larsson and Haux (1996) and Amcoff et al. (1998b and 1999a) described these broodfish salmon as wiggling due to their uncoordinated swimming. This type of behaviour has not been very common in Finland: The broodfish material of Lautiosaari State Fish Hatchery, Keminmaa, Finland comprising ca. 50-100 feral or wild Baltic salmon per year has included only ca. 10 fish displaying such behaviour since 1993 (J. Ryttilahti pers. comm. 2003). However, 3 wild adult Baltic salmon broodfish from the River Tornio sent by fishermen to the Regional Unit of Oulu of the National Veterinary and Food Research Institute, which is in charge of investigations of sick wild fish in northern Finland, from 1995-1999 had anamnesis of uncoordinated swimming and negative pathological and bacteriological findings. They could have been victims of thiamine deficiency disease, although the rapid post-mortem decrease in thiamine concentrations made a verified diagnosis impossible.

2.1.2. Behavioural disturbances

Mortality syndromes have clinical symptoms prior to death, which can be considered as abnormal behaviour. The term behavioural disturbance is used here to differentiate abnormal behaviour related to EMS from an actual, lethal mortality syndrome. Abnormal behaviour associated with thiamine deficiency disease has recently been reported in lake trout of the Great Lakes as causing problems but not death: Carvalho et al. (2002) noted reduced visual acuity in fry and Fitzsimons et al. (2002) described impaired predator avoidance and prey capture of larval lake trout with low
concentrations of thiamine, but not lethal thiamine deficiency. Not even all the wiggling Baltic salmon broodfish die before spawning (Börjeson et al., 1994).

2.2. M74 syndrome

2.2.1. Discovery of the syndrome, occurrence and effects on the reproduction of Baltic salmon

In 1974 Jonas Sahlin, the fishery manager of a fish farm at Bergeforsen on the River Indals, Sweden, observed abnormal behaviour in yolk-sac fry of Baltic salmon originating from wild broodfish and noted that some family groups had high mortality rates. He also gave the syndrome the designation M74 (Amcoff, 2000). Hansson et al. (2001) verified the lower annual mortalities at the salmon hatcheries of two Swedish Baltic salmon stocks in 1928-63. It is thus unlikely that M74 syndrome had occurred to a considerable extent before 1974. In Finland the incubation of wild Baltic salmon eggs was performed on batches of eggs originating from several females until 1985. In the following year a ban on the transportation of live fish came into force together with an obligation to examine the broodfish producing the eggs to be transported into inland waters (Ministry of Agriculture and Forestry, 1986). This led to the present system of separately incubating the eggs from each female (in order to enable the destruction of eggs from only those females in which a vertically transmissible infectious fish pathogen has been diagnosed). According to Soivio (1994), M74-like symptoms were observed in Finland in fry that hatched in the spring of 1987, but M74 syndrome was first positively diagnosed in 1992 (Koski et al., 1996; Soivio, 1996).

2.2.1.1. Occurrence of M74 in Finland

Monitoring of the occurrence of M74 in Finland has been conducted at the Finnish Game and Fisheries Research Institute on the basis of characteristic symptoms in the dying fish. Eggs for experimental incubation have been obtained from ascended spawners caught by State fish hatcheries. Eggs are incubated in the hatcheries or in the laboratory of the Finnish Game and Fisheries Research Institute in Helsinki. Mortality of the yolk-sac fry is recorded from hatching until the end of the yolk-sac phase. The monitoring results of salmon from the three rivers in Finland where it has been possible to catch enough wild or feral broodfish for this purpose are presented in Figure 2.
Figure 2. Annual prevalence of M74 syndrome in the fry of Baltic salmon of the Simo, Tornio and Kymi river strains. Black bars: The prevalence in feral broodfish females, the offspring of which suffered 100% mortality; White bars: The prevalence in feral broodfish females in whose offspring symptoms of M74 syndrome were seen; Gray bars: Mortality of yolk-sac fry. The figures were kindly provided by Finnish Game and Fisheries Research Institute.

The prevalence of M74 seemed to be higher in the material from the Bothnian Bay rivers Tornio and Simo than in the River Kymi flowing into the Gulf of Finland. The highest yolk-sac mortalities occurred around the mid-1990s, when ca. 60-80% of the annual offspring from the monitored females died. According to Soivio (1994), the cumulative yolk-sac fry mortality of the offspring from farmed broodfish kept at the State Fish Hatchery of Lautiosaari, Keminmaa, Finland in 1990 was 5.8% for River Simo and 7.9% for River Tornio salmon. The highest prevalences of M74 syndrome in Sweden also occurred in the 1990s. The peak mortality of 72% among females caught from the wild to supply compensatory hatcheries, which lost all their offspring, occurred in 1993 (Norrgren et al., 1998). M74 has not, however, been observed in wild or feral Baltic salmon from Latvia (Mitans, 1994) or Poland (Bartel, 1996).

2.2.1.2. Effects on the reproduction of Baltic salmon

There is no direct proof of M74 syndrome occurring in the yolk-sac fry of Baltic salmon populations in the wild salmon rivers. The probable effects on the reproduction and stocks in the wild have, however, been studied in Sweden and Finland. Karlström (1999) found a statistically significant negative correlation between M74 mortality in hatcheries and the parr/spawner ratio in three rivers flowing into the Bothnian Bay from Sweden. The result was a 63% reduction in the number of parr produced per ascending spawner. Karlström came to this conclusion by comparing the results of spawning in the severe M74 syndrome period from 1992-1996 (mean mortality 61%, range 52-74%) with those of years 1988-1991 and 1997, when mortality was lower (mean 15%, range 7-33%). A similar decrease in the natural parr production in the River Simo from 1992-95 was reported by Jokikokko et al. (1995). Romakkaniemi et al. (2003) suggested, however, that the
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effects of M74 syndrome on the stock of River Tornio salmon in the 1990s were counteracted by
dhery regulations and a good spawning stock in the worst M74 years.

2.2.2. Symptoms and findings

In the first published description of M74 syndrome, Norrgren et al. (1993) reported it as mortality of
whole offspring groups of certain wild Baltic salmon females over a period of less than one week,
when ca. 2/3 of the yolk-sac had been used. There are no reports of M74 occurring in farmed
Baltic salmon offspring. Lundström et al. (1998) differentiated between preclinical, clinical and
terminal stages of the disease and the same authors broadened the onset of the clinical disease to
occur in an early (326-359 day-degrees from fertilisation), intermediate (349-492 day-degrees) or
late (447-631 day-degrees) phase.

The death of the whole progeny of a salmon female was regarded as a characteristic of M74
syndrome. However, Amcoff et al. (1999a) reported M74 syndrome (diagnosed on the basis of
typical clinical symptoms and lesions) in the offspring of three females that resulted in mortality
under 100% and termed it partial M74. According to the statistics of the Finnish Game and
Fisheries Research Institute (Figure 2 in this thesis), this partial M74 has not been uncommon in
Finland. The role of male broodfish was regarded non-existent (Börjeson et al., 1994) in the
development of M74 syndrome in the fry. Females exhibiting “wiggling” behaviour before the
spawning time were reported to be predisposed to producing offspring with M74 syndrome
(Börjeson et al., 1994; Amcoff et al., 1996). M74 syndrome has also been reported to occur to a
minor extent in Baltic Sea trout (Amcoff et al., 1999a; Landergren et al., 1999).

The symptoms and lesions reported in the yolk-sac fry suffering from M74 syndrome are
summarised in Table 1.

Table 1. Summary of the diagnostic symptoms and lesions in salmon yolk-sac fry suffering
from M74 syndrome

<table>
<thead>
<tr>
<th>Symptom or lesion</th>
<th>Study with first report¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical symptoms</td>
<td></td>
</tr>
<tr>
<td>Sharp decrease/loss of avoidance reaction</td>
<td>a</td>
</tr>
<tr>
<td>Abnormal swimming, later inability to move</td>
<td>a</td>
</tr>
<tr>
<td>Changes in colouration of the skin</td>
<td>a, b, c</td>
</tr>
<tr>
<td>Bradycardia</td>
<td></td>
</tr>
<tr>
<td>Small and pale spleen</td>
<td>c</td>
</tr>
<tr>
<td>Exophthalmia</td>
<td></td>
</tr>
<tr>
<td>Haemorrhages, oedema and white precipitates in the yolk-sac</td>
<td>a</td>
</tr>
<tr>
<td>Delayed absorption of yolk</td>
<td>a</td>
</tr>
<tr>
<td>Pale staining of the yolk-sac droplet</td>
<td>c</td>
</tr>
<tr>
<td>Increased volume of the gall bladder</td>
<td>c</td>
</tr>
<tr>
<td>Biochemical abnormalities</td>
<td></td>
</tr>
<tr>
<td>Lower hepatic activities of two TPP-dependent enzymes, transketolase and α-</td>
<td>f</td>
</tr>
<tr>
<td>ketoglutarate dehydrogenase (α-KGDH)</td>
<td></td>
</tr>
<tr>
<td>Altered activity of the hepatic cytochrome P450 dependent 7-ethoxyresorufin-O-</td>
<td>a, d, g</td>
</tr>
<tr>
<td>deethylase (EROD)</td>
<td></td>
</tr>
<tr>
<td>Histopathological changes</td>
<td></td>
</tr>
<tr>
<td>Necrotic brain lesions</td>
<td>e</td>
</tr>
<tr>
<td>Glycogen depletion in the liver and skeletal muscle</td>
<td>a, e</td>
</tr>
</tbody>
</table>

¹: a = Norrgren et al. (1993), b = Börjeson et al. (1994), c = Lundström et al. (1998), d = Amcoff et al. (1999b), e =
Lundström et al. (1999a), f = Amcoff et al. (2000), g = Lundström et al. (1999b)
2.2.2.1. Clinical symptoms and gross lesions

The first observations of the clinical symptoms come from fish farmers, but Table 1 presents the reports in the scientific literature. The most detailed studies on the symptoms and gross lesions were those of Lundström et al. (1998 and 1999a), where clearly defined changes were graded and analysed in different stages or times of onset of the disease. The gross lesions were not all analysed macroscopically, but are included here, because some of them were performed with low magnification microscopy on living fry. For skin colouration, both paler and darker discoulorations have been reported, this dual possibility probably representing the darkening of the skin due to thiamine hypovitaminosis or lightening due to anaemia (see e.g. Christensen, 1966).

2.2.2.2. Biochemical disturbances

The literature concerning thiamine concentrations is reviewed in the chapter 2.2.3.1.

The activity of the TPP-dependent enzyme transketolase in different tissues has been used in the measurement of thiamine deficiency in fish for decades (Lehmitz and Spannhof, 1977; Masumoto et al., 1987). Amcoff et al. (2000) found that in Baltic salmon fry with M74 activities of both transketolase and another TPP-dependent enzyme, α-ketoglutarate dehydrogenase (α-KGDH) were lower than in healthy controls.

The changes in the activity of hepatic cytochrome P450 dependent enzyme EROD are confusing, because both increased Norrgren et al. (1993) and decreased (Amcoff et al., 1999b; Lundström et al., 1999b) activities have been reported. This may, however, be due to the limited duration of EROD activity in salmonid fish (Pesonen and Andersson, 1991; Amcoff et al., 1999b). The EROD measurement has several methodological questions to be resolved. Many physiological and environmental factors affect the cytochrome P450 system in fish (Sijm and Opperhuizen, 1989; Goksøyr and Förllin, 1992), which probably limits the use of the measurements of EROD activity as a diagnostic tool for M74 syndrome.

Lundström et al. (1999b) also reported higher activities of the hepatic enzymes glutathione peroxidase and glutathione reductase in the yolk-sac fry developing M74 than in healthy fry. They regarded this to show that the peroxidation mechanisms are involved in the pathogenesis of M74 in the yolk-sac fry of Baltic salmon. Newly-hatched fry that later developed M74 showed an induction of hepatic catalase activity. During the development of the fry, however, the situation reversed, with fry developing M74 failing to show increasing hepatic catalase activity, while this activity increased in healthy fry between hatching and the age of ca. 200 day-degrees.

In addition to the biochemical abnormalities in the fry, the females giving birth to M74 offspring were also shown to have biochemical disturbances in the description of M74 syndrome by Norrgren et al. (1993): their total hepatic cytochrome P450 content was higher than in females that produced normal yolk-sac fry.

Histopathological lesions

The brain lesions reported by Lundström et al. (1999a) differed in fry according to the stage and time of the onset of the disease. There was a high abundance of necrotic cells especially in the mesencephalon, diencephalon and cerebellum in clinical stage of the disease in fry with an intermediate or late onset of the disease. In the more aggressive M74 with an early onset there was also slight hydropic degeneration of the periventricular area and slight proliferation and hypertrophy of the capillaries of the brain stem. As the lesions progressed, there were also multifocal haemorrhages in the brain parenchyma and sometimes an increase in the hyaline content of the brain ventricules. In addition to the brain lesions and the glycogen depletion of the...
The occurrence and prevention of the M74 syndrome, a thiamine deficiency disease in Baltic salmon

hepatocytes and skeletal muscle (decreased intensity in periodic acid Schiff, PAS, staining),
histopathological differences of a lower grade between the M74 and healthy fry were also
observed in other organs. One of these, minor hepatocellular necrosis or hepatocyte degeneration
(and depletion of glycogen, too) was also found by Amcoff et al. (1999b) and Åkerman et al. (2003)
in Baltic salmon fry, where M74-like lesions were produced by the microinjection of thiamine
antagonists.

2.2.3. Aetiology

Possible genotype-dependent susceptibility to M74 syndrome was studied by Naevdal and Skaala
(2003), but they found no obvious connection between the mortality of the fry and allozyme
genotype or individual heterozygosity of the parent salmon. The role of infectious aetiology also
seems to be negligible (Cooray et al., 1999).

2.2.3.1. Thiamine deficiency

It is now generally accepted that the key factor in the development of M74 syndrome is the low
concentration of thiamine in the eggs and a subsequent thiamine deficiency in the yolk-sac fry (e.g.
Amcoff, 2000). The degenerative neuropathological lesions in M74 reviewed in chapter 2.2.2.3
resemble those described from thiamine deficiency diseases of mammals (Jubb and Huxtable,
1993). The beneficial effect of thiamine treatments in a dose-dependent manner (see chapter
2.2.4.) also strongly indicated the aetiological role of thiamine in the syndrome.

The concentrations of thiamine in the eggs and yolk-sac fry of the family groups that developed
M74 were low, often under half of those in the healthy family groups of the wild Baltic salmon in
Sweden in the mid-1990s. The mean (SD) concentration of the fertilised eggs of 15 females
producing M74 offspring was 1.1 (0.25) nmol/g, while it was 2.3 (0.86) nmol/g in 4 females
producing healthy yolk-sac fry. (The highest mean (SD) concentrations of the eyed egg groups
developing into M74 fry were 0.62 (0.13) nmol/g (N = 4) and the lowest of the healthy groups 1.1
(0.89) nmol/g (N=16).) The onset of the symptoms of M74 was also in correlation with the
concentration of thiamine in the eyed egg. Amcoff et al. (1999a) did not, however, report a
threshold value in the eggs, which could be used in the prognosis of M74. For yolk-sac fry, Amcoff
(2000) suggested a thiamine threshold limit interval of 0.31-0.58 nmol/g for the development of
M74. The tissue concentrations of thiamine in the adult fish ovary, white muscle and liver were also
shown to be lower in females producing M74 offspring or broodfish showing wiggling behaviour
than in controls. The concentrations in farmed Baltic salmon and those of the Swedish west coast
rivers were much higher. The concentrations in farmed broodfish (Amcoff et al., 1999a) are
reviewed in chapter 2.3.3.2.

Several possible factors leading to the low thiamine concentrations in the eggs have been
suggested and these will be reviewed in the following chapters.

2.2.3.2. Environmental toxins

The elevated activity of hepatic cytochrome P-450 in the female and yolk-sac fry and EROD
enzymes in the yolk-sac fry developing M74 (Norrgren et al., 1993) have led to intense
toxicological studies on the Baltic salmon. Both polychlorinated biphenyls (PCBs) and 2,3,7,8-
tetrachlorodibenzo-p-dioxin are examples of xenobiotics inducing the cytochrome P-450 system in
fish (Goksøyr and Förlin, 1992). Asplund et al. (1999) compared the concentrations of over one
hundred organohalogen substances (incl. PCBs) in the muscle, egg and blood of females
producing normal and M74 offspring and found no support for the hypothesis of these substances
are behind M74 syndrome. Vuorinen et al. (1997) reported, however, a correlation between certain
dioxin-like organochlorines (co-planar PCBs and polychlorinated dibenzofuran (PCDF) congeners)
and the prevalence of M74 mortality in River Simo salmon in the years 1989-1993. The
pathological lesions reported in the fry of lake trout (Spitsbergen et al., 1991) and rainbow trout
(Helder, 1981) exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin were, however, different from the lesions reported in M74 (chapter 2.2.2.) and Cayuga syndrome (Fisher et al., 1995). In the experimental study of Amcoff et al. (2002a), an association between the whole body thiamine concentration and hepatic EROD activity was reported, which is in accordance with two earlier reports from yolk-sac fry developing M74 in Sweden (Amcoff et al., 1999b; Lundström et al., 1999b), but in contrast to the findings of Norrgren et al. (1993). In addition to dioxin like environmental toxins (Goksøyr and Förlin, 1992), astaxanthine (Gradelet et al., 1996) has also been reported to be a strong inducer of liver EROD activity in rat.

### 2.2.3.3. Other aetiological factors concerning nutrition

Pettersson and Lignell (1999) found a correlation between the astaxanthin concentrations of the eggs and the development of M74 in yolk-sac fry. They reported that all the egg batches having an astaxanthin concentration above 0.22 µg/egg gave rise to healthy yolk-sac fry, but eggs with concentrations below 0.15 µg/egg all gave rise to M74 yolk-sac fry. In the range 0.15-0.22 µg/egg, both healthy fry and those with partial M74 (mortality 50-60%) were observed. In addition to the lower concentrations of astaxanthine, a lower astaxanthine/total carotenoid ratio in the eggs developing to M74 fry has also been detected. The latter finding was not considered to be caused by an elevated consumption of carotenoids due to oxidative stress, but rather by a limited availability of astaxanthine in the feed of Baltic salmon (Lundström et al., 1999b). The orange colour of certain tissues of salmon is derived from carotenoid pigments (Hardy, 1989) in their diets, the most important of which in the egg is astaxanthin (Pettersson and Lignell, 1999). During sexual maturation there is a mobilisation of the carotenoids from the muscle into the developing eggs and the skin (Torrissen, 1989). In the past it was hypothesised that carotenoids would protect the developing egg from light, but the experiments reported by Torrissen (1989) do not support this. Pettersson and Lignell (1999) mentioned roles as a precursor of retinols and a source of intracellular oxygen reserves as possible functions of carotenoids in fish and hypothesised the long period of embryonic development in salmon to impose an extra high demand for oxidative protection.

The amount of the absorbable thiamine in the gastrointestinal canal can be influenced by many dietary factors in addition to the amount of thiamine in the diet per se. Probably the most important one is the existence of thiamine destroying enzymes, thiaminases, in the diet. The Cayuga syndrome in the land-locked Atlantic salmon of certain New York Finger Lakes has been shown to be caused by a diet rich in a prey fish species, alewife (*Alosa pseudoharengus* (Wilson)), that has a high thiaminase activity (Fisher et al., 1996). Thiaminase-containing diets of farmed salmonid species have long been known to cause thiamine deficiency in the on-growing fish at fish farms (Christensen, 1966; Saunders and Henderson, 1994). The gastrointestinal canal (excl. the posterior intestine) was shown to have high thiaminase activity in the adult Baltic salmon caught during their spawning migration towards north from the Baltic Proper in the Gulf of Bothnia (Wistbacka et al., 2002). The potential thiaminase activity was even higher than the mean activity of the local herring, which led the authors to speculate about the possibility of Baltic salmon feeding selectively on Baltic herring with high thiaminase activity. However, the spatial, seasonal and species specific (especially those of herring (*Clupea harengus* L.) and sprat (*Sprattus sprattus* (L.))) variation in thiaminase activity (Soivio and Hartikainen, 1999; Wistbacka et al., 2002) and its association with the variation in the diet of Baltic salmon (chapter 2.4.2, Koski, 1999) needs much more research to obtain a better understanding of the maternal dietary factors behind the thiamine deficiency of yolk-sac fry developing M74 syndrome.

### 2.2.3.4. Other factors affecting the requirement and supply of thiamine in the female broodfish of Baltic salmon

The different stocks of Baltic salmon have wide variety in their migration routes between the feeding areas and the spawning rivers (Figure 4), the longest being those of the stocks of the
Bothnian Bay rivers. According to Christensen and Larsson (1979), during the long spawning migration covering many miles each day, the salmon does not eat as much as in the feeding areas (chapter 2.4.2.), but is probably at least as metabolically active as when feeding in the Baltic proper. The more northerly the river the earlier is the spawning run into the river (Christensen and Larsson, 1979), which can further increase the requirement for thiamine in the feeding areas to guarantee the needs of the future offspring. The broodfish of the Bothnian Bay rivers reduce their feeding during the migration towards the spawning river long before arriving at the river mouth, but there are indications that the Latvian salmon in the Riga Bay feed later in the summer (Karlsson et al., 1999). There are also differences in the prey fish composition of the salmon, for example in the Riga Bay and the Gulf of Bothnia, before entering the spawning river (chapter 2.4.2.). Herring and sprat in the diet without additional thiamine has been known to lead to thiamine deficiency in the rainbow trout, but marine fishes like small sandeels (Ammodites tobianus L.) could be given without the need for addition of the vitamin (Christensen, 1966).

2.2.4. Treatment and prevention

The potential of thiamine bathings to cure EMS in lake trout and Cayuga syndrome in Atlantic salmon was discovered in America in the mid-1990s (Fitzsimons, 1995; Fisher et al., 1996). Experiments to prevent the development of M74 syndrome in the fry by dietary manipulation of the female were begun in Sweden before this (Börjeson et al., 1999), but Bylund and Lerche (1995) were the first to try thiamine treatment for the therapy and prevention of Baltic salmon fry showing M74-like symptoms. They found preventive treatments with 800 mg/l or 8000 mg/l TH-HCl for 30 or 60 minutes to prevent high mortality of the yolk-sac fry originating from one Baltic salmon female and cure the symptoms of the control group, when administered during the symptoms. More comprehensive immersion treatments of the fry and eggs and injection of the females have since also been performed. These trials have been summarised in Table 2.

Table 2. Reported treatments with thiamine hydrochloride for the therapy (T) and prevention (P) of M74 syndrome in Baltic salmon

<table>
<thead>
<tr>
<th>Intention of the treatment (T/P)</th>
<th>Treated fish</th>
<th>Time of treatment</th>
<th>Route of administration¹</th>
<th>Dosage</th>
<th>Additions</th>
<th>Reported result</th>
<th>Study²</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/P</td>
<td>Stripped females</td>
<td>During following summer(s)</td>
<td>p.o.</td>
<td>Normal pelleted feed according to appetite</td>
<td>Force feeding or mixing the salmon with rainbow trout</td>
<td>Increase in egg thiamine concentrations; lower M74 prevalence than before reconditioning</td>
<td>d</td>
</tr>
<tr>
<td>T &amp; P</td>
<td>Yolk-sac fry before and with M74-like symptoms</td>
<td>640 day-degrees after fertilisation and during the symptoms</td>
<td>Immersion</td>
<td>800 and 8000 mg/l for 30 and 60 minutes</td>
<td>pH adjustment; supplementary oxygenation of water</td>
<td>Recovery from the symptoms and prevention of mortality in the preventively treated</td>
<td>a</td>
</tr>
<tr>
<td>P</td>
<td>Yolk-sac fry before clinical disease</td>
<td>282-383 day-degrees post fertilisation</td>
<td>Immersion</td>
<td>0, 500, 2500 and 12500 mg/l for 1 hour</td>
<td>pH adjustment; supplementary oxygenation of water</td>
<td>Dose dependent prevention of M74</td>
<td>b</td>
</tr>
</tbody>
</table>
The efficacy of treatments has been good or excellent, but repeated treatments when using the immersion method have been reported to be needed in practice (Amcoff et al., 1998a). No side-effects have been reported.

No drugs other than thiamine hydrochloride have been reported in the treatment of M74 syndrome. In America, several compounds other than thiamine have, however, been tested in the treatment of EMS and Cayuga syndrome: In his trials, which led to the discovery of the effect of thiamine in EMS of lake trout, Fitzsimons (1995) also tested other vitamins of the B group (nicotinic acid, riboflavin, folic acid and pyridoxine), but found no beneficial response. Hornung et al. (1998) reported a stage specific effect of thyroxin immersion on EMS of newly-hatched steelhead trout, but no effect of carotenoid injections into the eggs. Fisher et al. (1996) injected vitamins A, C and E into the yolk-sac fry of Atlantic salmon of the Cayuga Lake without alleviation of the mortality due to Cayuga syndrome.

2.3. Thiamine and its metabolism in animals

2.3.1. Chemistry of thiamine

Thiamine is a water soluble vitamin of the B group (vitamin B₁). The molecular weights of the different forms (free base, TH; thiamine monophosphate, TMP; thiamine pyrophosphate, TPP; thiamine triphosphate, TTP and thiamine hydrochloride, TH-HCl) differ markedly from each other (Table 3). The solubility of thiamine in water is one in one, while that in alcohol is 1 in 100 (Dollery, 1991).
The occurrence and prevention of the M74 syndrome, a thiamine deficiency disease in Baltic salmon

Table 3. Abbreviations, molecular weights and the conversion coefficients between amounts in different units of the different forms of thiamine

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Form of thiamine</th>
<th>Molecular weight (g/mol)</th>
<th>1 µg/g equals (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td>Free thiamine</td>
<td>300.8</td>
<td>3.32</td>
</tr>
<tr>
<td>TMP</td>
<td>Thiamine monophosphate</td>
<td>380.0</td>
<td>2.63</td>
</tr>
<tr>
<td>TPP</td>
<td>Thiamine pyrophosphate</td>
<td>460.0</td>
<td>2.17</td>
</tr>
<tr>
<td>TTP</td>
<td>Thiamine triphosphate</td>
<td>539.9</td>
<td>1.85</td>
</tr>
<tr>
<td>TH-HCl</td>
<td>Thiamine hydrochloride</td>
<td>337.3</td>
<td>2.96</td>
</tr>
</tbody>
</table>

Thiamine is composed of pyrimidine and thiazole moieties linked by a methylene bridge. The reactivity of the coenzyme TPP centres on the carbon-2 atom of its thiazole ring. The other regions of the TPP molecule probably act as “hafts”, by which the enzyme holds the coenzyme in the correct position (Figure 3).

![Figure 3](image-url)

Thiamine diphosphate as a coenzyme in an enzyme. Different parts and the reactive centre of the molecule. Drawing based on Figure 3-47 of Alberts et al. (1983), with permission of the publisher.
2.3.2. Thiamine in the vertebrate cellular metabolism

The active form of thiamine in a vertebrate cell is TPP. It is required to derive pyruvic acid from either glycolysis or amino acids to enter the citric acid cycle via acetyl CoA. The multienzymic dehydrogenase complexes, for example pyruvate dehydrogenase, are localised in the mitochondria (McGormick and Greene, 1994). TPP is also a coenzyme for the transketolase in the pentose pathway of hexose metabolism. Transketolase is found in the cytosol of many tissues, especially liver and blood cells, in which principal carbohydrate pathways exist (McGormick and Greene, 1994).

Besides its important role in energy metabolism, TPP also takes part in decarboxylation in the synthesis of the transmitter acetylcholine, which carries a nerve impulse from one nerve cell to the next (Reed, 1980). In the nervous system thiamine has other roles to that of TTP: it regulates large conductance chloride channels, serves as a phosphate donor and modifies ion transport, especially sodium (see Gibson and Zhang, 2002).

2.3.3. Metabolism of thiamine in salmon

Much of the research on the metabolism of thiamine has been performed in mammals. In order to obtain a comprehensive picture the metabolism in mammals is shortly reviewed here in the description of the metabolism of thiamine in salmon. Discussion of physiological factors that may influence the metabolism of thiamine in salmon is also included.

Absorption

Thiamine is well absorbed from the upper small intestine in man (Dollery, 1991). The process is active in low and physiological ranges of dietary thiamine (5 mg daily in man). In high and pharmacological dietary concentrations the absorption is mainly by passive diffusion (McGormick and Greene, 1994; Rindi and Laforenza, 2000). The active and passive uptake mechanisms are common in mammals (Bowman et al., 1989), but the type of the absorption in fish has not been reported.

Transport, metabolism and distribution

In the active transport of the extracellular free thiamine (TH) into the cells, two thiamine transporters have been described: THTR-1 (Dutta et al., 1999; Subramanian et al., 2003) and THTR-2 (Rajcobal et al., 2001). Thiamine monophosphate (TMP) may be transported into the cell in certain tissues by a reduced folate carrier (Zhao et al., 2002). The role of different transporters in different tissues is still largely unknown, but THTR-1 is highly expressed in skeletal muscle (Zhao et al., 2002). Thiamine transport activity in mammals has been functionally characterised in several cell types including erythrocytes (Casirola et al., 1990) and hepatocytes (Chen, 1978). The types of absorption in fish have not been reported. The phosphorylation of ingested TH into TPP takes place in the enterocyte in mammals. Exit from the enterocyte is directly dependent on the activity of Na⁺-K⁺-ATPase (Rindi and Laforenza, 2000). These authors summarised the present knowledge of the transcellular thiamine transport in the enterocyte in a figure that is reproduced in Figure 4.
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Figure 4. Transcellular thiamine transport by the rat enterocyte according to Rindi and Laforenza (2000, reproduced with the permission of the publisher). The entry of T+ is largely by exchange with H+ (A) and very little occurs by enzymatic transphosphorylation to TMP. Cellular crossing is associated with intracellular enzymatic phosphorylation to TPP. The exit is directly dependent on the activity of Na⁺-K⁺-ATPase (C). The numerical values are approximate. TPK: thiamine pyrophosphokinase; TMPase, TPAs: thiamine mono-, pyrophosphatase; A: T+ / H+ antiport; B: Na⁺ / H+ antiport.

TH occurs in the plasma, but TPP predominates in the cellular components in man. Brown et al. (1998) reported a corresponding distribution in a small number of lake trout samples: in the eggs and plasma the major form was TH, but thiamine in erythrocytes, liver and kidney was in the form of TPP. The rapid intracellular phosphorylation of TH to TPP is behind the increase in the volume of distribution \( V_d \) from the initial value of approximately equal to the extracellular space to one much larger than the body water volume in man, when large parenteral thiamine doses are administered. The charged TPP is trapped intracellularly (Weber et al., 1990). Gold et al. (1995) reported over ten-fold concentrations of thiamine in the erythrocytes compared to those in plasma in healthy humans. Of the ca. 30 mg thiamine stored in the adult human body, 80% occurs as TPP, 10% as TTP and the rest as TH and TMP (McGormick and Greene, 1994).

The formations of the phosphorylated forms of thiamine are catalysed by the following enzymes:
- thiaminokinase (=thiamine pyrophosphokinase): formation of TPP and AMP from TH and ATP
- TPP-ATP phosphoryl-transferase: formation of TTP and ADP from TPP and ATP
- thiamine diphosphatase: dephosphorylation of TTP to TMP
- thiamine triphosphatase: hydrolysis of TTP to TPP

Thiaminokinase is widespread, but the others are mainly found in the nervous tissue (McGormick and Greene, 1994; Gibson and Zhang, 2002).

According to McGormick and Greene (1994), about half of the body stores of thiamine in man are found in skeletal muscles, with much of the remainder in heart, liver, kidneys, and nervous tissue (including the brain, which contains most of the TTP). The body stores of thiamine in fish have not been studied, but concentrations in different tissues vary: Brækkan (1959) reported thiamine values of 1.3 µg g-1 fresh weight in white muscle, 4.3 µg g-1 in red muscle and 4.0 µg g-1 in the liver of wild Norwegian Atlantic salmon. Sautier (1946, cited in Niimi et al. (1997)) found the highest concentrations in the eggs (2-4 µg g-1), 1-2 µg g-1 in the liver and ca. 1 µg g-1 in the muscle of
five species of wild Pacific salmon. The concentrations reported by Amcoff et al. (1999a) also vary between tissues. The highest mean values in farmed adult female Baltic salmon were obtained from ripened ovaries and lowest from intestines sampled behind the pyloric caeci. The mean thiamine concentrations in different tissues were as follows (mean (SD), conversion of units as TH (Table 3):

- ovaries 5.4 (0.9) µg g⁻¹
- red muscle 5.1 (0.6) µg g⁻¹
- liver 4.5 (1.5) µg g⁻¹
- head kidney 4.5 (1.5) µg g⁻¹
- heart 3.6 (0.2) µg g⁻¹
- main kidneys 3.6 (0.8) µg g⁻¹
- brain 3.3 (0.2) µg g⁻¹
- white muscle 2.0 (0.5) µg g⁻¹
- intestine 1.3 (0.2) µg g⁻¹

Malyarevskaya and Karasina (1991) found seasonal variation in the thiamine concentrations of roach (Rutilus rutilus (L.)) and pike (Esox lucius L.), the highest concentrations occurring in prespawning fish.

The diffusion of water-soluble substances such as thiamine into the brain is restricted because of the blood-brain barrier (Benet et al., 1996), and absorption across the barrier in the rat involves similar mechanisms to that in the intestine (Haas, 1988).

Catabolism, excretion and toxicology

Thiamine is filtered by the glomerulus as TH or metabolites (at least 20 metabolites have been found in rat and human urine, one of the quantitatively important ones being pyrimidine carboxylic acid). The excretion of pyrimidine and thiazole moieties may even increase when intact thiamine is no longer measurable in urine. These are considered to represent thiamine catabolised by the tissues and therefore the loss of body stores of thiamine (Lamden, 1972). At high doses of intravenous thiamine hydrochloride there is dose dependent nonrenal clearance in man. At low plasma concentrations, tubular reabsorption occurs (Weber et al., 1990).

According to Unna (1972), very large intravenous doses are needed to cause death in animals, e.g. 125 µg g⁻¹ in mice and 350 µg g⁻¹ in dogs, which is due to depression of the respiratory centre. Rapid intravenous injections of 5-50 µg g⁻¹, however, cause a transient dose dependent fall in blood pressure in cats and dogs. No toxic effects of thiamine in fish have been published.

Requirements

The requirement for thiamine is dependent on the carbohydrate intake and the metabolic rate of an animal. According to Halver (1989), the requirement for carnivorous fishes is not much different from the National Research Council (1993) estimate of 0.5 mg/1000 kcal dietary intake for mammals. The requirement was, however, considered to be only ca. 0.02 mg/1000 kcal digestible energy by (Morito et al., 1986). In some herbivorous mammals the microflora of the alimentary canal is also important in synthesising thiamine (Jansen, 1972; Dollery, 1991; McGormick and Greene, 1994). Such activity was reported in the intestine of lake trout by Ji et al. (1998), but Cooray et al. (1999) found no evidence of such bacteria in their study of the microflora in the gastrointestinal canal of five male spawners of Baltic salmon. A summary of the requirement for thiamine and deficiency signs or illnesses of certain nonsalmonid farmed fish and other animals including man is provided in Table 4.
The occurrence and prevention of the M74 syndrome, a thiamine deficiency disease in Baltic salmon

Table 4. Thiamine requirements and deficiency signs or illnesses of certain nonsalmonids, farmed animals and man

<table>
<thead>
<tr>
<th>Species</th>
<th>Age/size</th>
<th>Requirement of thiamine in the diet</th>
<th>Deficiency signs/illness</th>
<th>Reference of the value for requirement¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel catfish (Ictalurus punctatus R.)</td>
<td>6-9 g</td>
<td>1 mg/kg dry diet</td>
<td>Loss of appetite, dark discoloration, increased mortality</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Growing</td>
<td>1-3 mg/kg (25 °C)</td>
<td></td>
<td>b</td>
</tr>
<tr>
<td>Turbot (Scophthalmus maximus (L.))</td>
<td>Growing</td>
<td>0.6-2.6 mg/kg dry diet</td>
<td>Cessation of growth</td>
<td>c</td>
</tr>
<tr>
<td>Carp (Cyprinus carpio (L.))</td>
<td>Growing</td>
<td>2-3 mg/kg dry diet (30 °C)²</td>
<td>Skin congestion, subcutaneous haemorrhage</td>
<td>b</td>
</tr>
<tr>
<td>Mink and foxes</td>
<td>All</td>
<td>Addition of 1-2 mg for mink and 2-3 mg for foxes into the daily feed</td>
<td>General disturbance of metabolism incl. development of embryos, Chastek paralysis</td>
<td>d</td>
</tr>
<tr>
<td>Cow</td>
<td>Calf</td>
<td></td>
<td>Cerebrocortical necrosis</td>
<td>e</td>
</tr>
<tr>
<td>Human</td>
<td>All</td>
<td>0.4-1.4 mg/day (depending on age and sex)</td>
<td>Beri-beri, Wernicke-Korsakoff syndrome</td>
<td>f</td>
</tr>
</tbody>
</table>

²: Or less, depending on the carbohydrate content of the diet.

In fish the metabolic rate is mainly dependent on the water temperature and the activity and size of the fish. The higher the temperature, the more a fish performs muscular work, while the smaller a fish is, the higher is the metabolic rate (Smith, 1982) and the greater the requirement for thiamine. The experiments on which the suggested thiamine requirements of salmonid fish are based have been performed on growing fish. This data is summarised in Table 5. The thiamine content of developing eggs of rainbow trout decreased by approximately half from ca. 0.44 µg in the unfertilised egg to the level at hatching (Sato et al., 1987). The requirement of the yolk-sac fry of salmonids has not been determined, but Mæland (2000) suggested that the requirement for water soluble vitamins including thiamine in hallibut (Hippoglossus hippoglossus L.) larvae may be higher than suggested for adults and juveniles of several cold-water teleost species by the National Research Council (1993).

According to Jansen (1972), the “thiamine-sparing” action of fats and proteins in the diet may depress the thiamine requirement of an animal to practically zero. The salmon broodfish mainly uses body lipid reserves as a source of nutrition during the spawning migration and the stay in the river (Jonsson and Hansen, 1997). This can be speculated to lower the thiamine requirements of wild salmon broodfish in comparison with farmed ones. It must not be forgotten, however, that the thiamine needs in the maturing eggs are considerable in both starving and fed broodfish.
Table 5. Thiamine requirements of salmonid fishes

<table>
<thead>
<tr>
<th>Species</th>
<th>Water temperature (ºC)</th>
<th>Age/size</th>
<th>Requirement for thiamine in the diet</th>
<th>Reference for the requirement value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout</td>
<td>15</td>
<td>0.5-2.2 g</td>
<td>1 mg kg⁻¹ dry feed</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>15 growing</td>
<td>22-40 g</td>
<td>10-12 mg kg⁻¹ dry feed</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>14-19</td>
<td></td>
<td>4 mg kg⁻¹ feed</td>
<td>c</td>
</tr>
<tr>
<td>Chinook and silver</td>
<td>10</td>
<td>growing</td>
<td>10-15 mg kg⁻¹ dry feed</td>
<td>d</td>
</tr>
<tr>
<td>salmon</td>
<td></td>
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</table>

¹: a = Morito et al. (1986); b = Phillips et al. (1946, cited in Halver (1989));
c = Jürss & Tiemann (1976); d = Halver (1989).

The difference in the requirements proposed in the earlier studies cited by Halver (1989) and that proposed by Morito et al. (1986) are great. This difference is further accentuated by the fact that the requirement proposed by the latter was based on tissue transketolase activity, which is a more sensitive indicator of deficiency than, for example, weight gain (Tiemann and Lehmitz, 1975), which was used in the earlier works cited by Halver (1989). The higher proposals for the requirements of salmonoids were heavily criticized by Woodward (1994). The reference work National Research Council (1993) points out a need for professional judgement in the selection of the best requirement value in each case and presents a wide range of 1-15 mg kg⁻¹ feed for salmonid species.

The enzymatic destruction of thiamine by thiaminases of the prey fish of landlocked Atlantic salmon in the Finger Lakes in USA has been shown to be the cause of Cayuga syndrome, a thiamine deficiency disease (Fisher et al., 1996). The thiaminase activity in the diet obviously affects the amount of available thiamine in the diet. The role of this and the possible other diet-related factors in the Baltic salmon female broodfish were reviewed in chapter 2.2.3.4.

2.3.4. Thiamine in the water ecosystem

The synthesis of thiamine from its pyrimidine and thiazole components was originally studied in yeasts (Brown, 1972). Microbes and microalgae are the primary sources of thiamine in aquatic ecosystems, too. Some algae are, however, thiamine-requiring and may use the thiamine excreted by the former organisms (Niimi et al., 1997). The concentrations of thiamine in filtered surface freshwater have been in the low ng l⁻¹ range and can be higher in small, eutrophic lakes. Levels in seawater have been found to be of a similar magnitude and higher in shallow and nearshore waters than deep and offshore ones. Ranges of values were from 10 ng l⁻¹ to 490 ng l⁻¹ (Niimi et al., 1997).

Field observations of waterborne thiamine appear to be related to phytoplankton productivity and increases in thiamine concentrations have been correlated with blooms and the success of algal populations. Thiamine is required as an essential nutrient by some members of the lower taxonomic groups and is generally required by all higher taxonomic groups. The direct uptake of thiamine from water can be an important pathway for lower trophic level organisms, but the diet is probably more important among those at higher trophic levels (Niimi et al., 1997). The dietary requirements of farmed prawns have been suggested to be rather high, 120 µg g⁻¹ diet for Penaeus japonicus and 14 µg g⁻¹ diet for Penaeus monodon, and for maximum growth of a farmed mollusc species, abalone (Haliotis discus hannai Ino), 51 µg g⁻¹ diet (Zhu et al., 2002).
2.4. Life of the Baltic salmon

2.4.1. Life cycle

Salmon in the Gulf of Bothnia and the River Teno have had to adapt their life cycles to a very cold environment. This has resulted in surprising speed in egg and yolk-sac development. In the study of Hamor and Garside (1976), the number of day-degrees needed for the development of Atlantic salmon eggs to the advanced prehatch stage 18 (schedule given by Garside (1959), cited in Hamor and Garside (1976)) was ca. 415 day-degrees at +5 °C and 430 day-degrees at +10 °C in water that was fully saturated with oxygen. Plomann and Vogel (1987) reported the number of day-degrees needed from the fertilisation to the hatching in Atlantic salmon to be 440. The development of salmon eggs at the low winter water temperature in Finland is much quicker: Heinimaa and Heinimaa (2004) reported the hatching of River Teno salmon to occur after only 181 ± 15 (mean ± SD) day-degrees. The hatching of Bothnian Bay salmon at the normal river temperatures occurs in May-June (unpublished statistics of the Lautiosaari State Fish Hatchery, Keminmaa, Finland). Lundström et al. (1998) reported the hatching to have occurred ca. 170 day-degrees from fertilisation in their study of Dal River Baltic salmon from the southern Gulf of Bothnia in Sweden.

During the first days of their life, fry hide among the gravel of the river bed in nature and thrive on the bottom of the hatchery trough of fish farms without eating. After the yolk-sac has been used, the fry rises from the bottom of the river (emergence, “swim-up”) and begins to take external food. Brännäs (1988) performed experiments on River Ume salmon and found that the number of day-degrees from hatching to the emergence was not constant, but increased with decreasing temperature (being e.g. 281 day-degrees at 12.2 °C and 441 at 6.6 °C). The rate of development from hatching to emergence in Baltic salmon (ca. 30 days in 10 °C) was twice that in data presented from the south of Canada (ca. 60 days at 10 °C). According to Lindroth’s (1950, cited in Christensen and Larsson, 1979) hypothetical survival analysis, the mortality of the wild salmon of the Swedish Botnian Bay rivers is ca. 20% during the first winter and over 90% during the first year. In fish farming the developing salmon face much fewer dangers and mortality is lower.

There is wide variation in the length of the parr stage in the different salmon stocks studied here. Young salmon usually spend 3 years in the rivers flowing into the Bothnian Bay (Järvi, 1938). In warmer rivers of the Baltic Sea, 1-2 years in the river are typical e.g. the most prevalent age-class of the smolt is 2 years in the Latvian River Salatsa and 1 year in the Swedish River Mörrum (Christensen and Larsson, 1979; Mitans, 1994). Among Atlantic salmon in the River Teno, the smolt age varies considerably, from 2-7 years, but mostly from 3 to 5 years (Erkinaro et al., 1998). At Finnish fish farms the smolt production has a goal of producing two-year-old smolts for stocking into the river mouths.

The feeding migration of the salmon differs between rivers of the Baltic Sea catchment area: salmon from the Bothnian Bay make the longest feeding migration to the southern part of the Baltic Proper, while Neva salmon stocked into the Gulf of Finland mostly remain in the Gulf of Finland, with some also migrating to the eastern part of the Baltic Proper, which are the primary feeding areas of Latvian salmon (Karlsson et al. 1996; 1999, Figure 5). The feeding migration of salmon from the Finnish Bothnian Bay rivers usually lasts 3 years, but 2 or 4 years at sea are also fairly common. There is also a strong grilse population (almost exclusively male fish spending only one year in the sea before migrating up the spawning river) among the Bothnian Bay salmon (Järvi, 1938; Kallio and Pruuki, 1987; Jokikokko and Jutila, 1998). The large material gathered before the building of hydroelectric power plants at the mouths of the former salmon rivers Kemi, li and Oulu shows that 95.1% of the spawning salmon population spawned only once, 4.4% twice, 0.4% three times, 0.03% four times and only one fish out of 23852 (0.004%) five times in their life (Järvi, 1938).
Figure 5. The feeding migrations (arrows) of the multi-sea-winter stocks of three salmon populations of the Baltic Sea: salmon from the Bothnian Bay, Gulf of Finland and Latvia (based on Karlsson & al 1999 and 1996). The present (thick lines) and past (thin lines) salmon rivers are also shown (from HELCOM, 1996).

The spawning migration of the “multi-sea-winter stock” (MSW), from the Baltic Proper starts in late March or in April. Their migration up the spawning rivers of the Bothnian Bay begins in the latter half of June and is mainly completed at the end of June (Christensen and Larsson 1979; Kallio and Pruuki, 1987). Atlantic salmon stop eating before they enter the spawning river and do not eat during their time in the river (Kadri et al., 1995). The salmon from the Bothnian Bay rivers spawn - or the broodfish are stripped in order to obtain eggs - usually in October and sometimes at the beginning of November (Brännås, 1988); unpublished statistics of the State Fish Hatchery of Lautiosaari, Keminmaa, Finland). The so-called autumn or winter salmon (females with weakly developed gonads spending the time from the autumn or winter prior to the spawning autumn in the spawning river), which are not uncommon in the rivers flowing into the Barents Sea and White Sea (Berg, 1962), are not known in Baltic salmon except the former salmon rivers Vistula and Helgeå (Christensen and Larsson, 1979; Bartel, 1994).
2.4.2. Diet of wild Baltic salmon

During the fry and parr phase the food of young salmon consists of aquatic animals that drift along the water current in the river: insects of different development stages, but also planktonic crustaceans and later in river life larger crustaceans and molluscs (Chistensen and Larsson, 1979; Erkinaro et al., 1995). Airborne food, mainly insects, may also be of importance (Christensen and Larsson, 1979). After arrival at sea the salmon of the Bothnian Bay rivers switch to eating terrestrial insects and fish instead of the food items in the river (Jutila and Toivonen, 1985). The diet of the postsmolts may vary among different parts of the Baltic Sea (Christensen and Larsson, 1979; Salminen et al., 2000).

Research into the diet of Baltic salmon (analysis of stomach contents) during its feeding migration in the 1957-1964 was summarised by Christensen and Larsson (1979). Contents of the average stomach had a large seasonal variation, the overall fullness in the winter being less than half of that in autumn and spring samples. Karlsson et al. (1999) performed a new study in 1995-97 that did not reveal such an obvious difference between seasons in the stomach fullness in the material of Baltic Proper. In both the previous studies and that of the years of M74 syndrome, the diet of salmon in offshore areas of the Baltic Proper was dominated by sprat and herring. A third frequently occurring prey was the three-spined stickleback (Gasterosteus aculeatus L.). The composition of the diet (in proportion of wet weight) of feeding salmon in the Baltic Proper in these studies is illustrated in Figure 6.

![Figure 6: The composition of the diet (in proportion of wet weight) of feeding salmon in the Baltic proper. The figure was obtained from Karlsson et al. (1999), with the permission of the author and the publisher. The symbol (less than) was corrected by the present author.](image-url)

Karlsson et al. (1999) suggested that there were stronger seasonal diet differences than the difference between the eastern and southern Baltic Proper. The proportion of sprat in the stomach contents was highest during the months January-April in comparison with the periods of May-September and even more with October-December. During the spawning migration in the Gulf of Bothnia (incl. Bothnian Bay) and in the Gulf of Riga, the salmon often had empty stomachs and in addition the dietary composition differed markedly from that of the feeding in the Baltic Proper (Karlsson et al., 1999). Sprat was absent from the stomachs of the salmon caught in the Gulf of Bothnia and herring comprised more than 90% of the stomach contents. In the Gulf of Riga there
was a total absence of sprat. “Other fish” (such as sand eels, perch (*Perca fluviatilis* (L.)), smelt (*Osmerus eperlanus* (L.)) and cod (*Gadus morhua* L.)) together with herring and three-spined stickleback constituted the diet.

### 2.5. Metabolism and pharmacokinetics of salmonids

#### 2.5.1. Metabolic rate

The metabolism of poikilothermic animals like the salmonids depends on the temperature of their environment. The metabolic rate is usually measured in terms of the consumption of oxygen. It can be described by the general equation (see e.g. Smith, 1982):

\[
10\log Q = a \cdot 10\log W + b \cdot 10\log t + c,
\]

where \(Q = \) consumption of oxygen/weight of fish/time (e.g. mg O\(_2\)/kg/h), \(W = \) weight of fish, \(t = \) temperature of the water and \(a, b\) and \(c\) are constants.

It can be concluded that the weight-specific oxygen consumption is higher in small than large fish. The effect of the temperature is, however, much more important and follows the general rule of a doubling of the metabolic rate with a rise in water temperature of 10 ºC. The overall metabolic rate in salmonids is low and even when close to maximum activity is only on the level of the basic metabolic rate of mammals (Smith, 1982).

One way to take into account the effect of the temperature on metabolism is through the use of day-degrees, which is the sum of the mean daily water temperatures, e.g. 300 day-degrees means 30 days at a water temperature of 10 ºC and 20 days at 15 ºC.

Several biotic and abiotic factors in addition to water temperature influence the metabolic rate, weight and level of activity of fish (Smith, 1982). When different phases of the life cycle are considered, at the end of the yolk-sac stage the relative oxygen demand of Baltic salmon is higher than at any other stage (Christensen and Larsson, 1979). Salmon starve during the upstream migration and spawning, using more than 60% of their total available energy (Jonsson and Hansen, 1997), and starvation is known to decrease the metabolic rate (Fry, 1957; Smith, 1982).

#### 2.5.2. Pharmacokinetics

The application of pharmacokinetics allows the processes of absorption, distribution and elimination of a chemical in an animal to be described mathematically. There is, however, relatively little information on the pharmacokinetics of chemicals in fish (Barron et al., 1990), and the pharmacokinetics of thiamine in fish has not been studied.

A pharmacokinetic study is often based on a bolus dose of a drug and the measurement of the drug concentrations prior to dosing and at subsequent sampling times in the serum, plasma or whole blood. The concentration of the drug is usually presented on a logarithmic scale on the y-axis and a time-concentration curves such as those in Figure 7 are obtained (see e.g. Birkett, 1998). This reference is used in the following paragraph in the presentation of some major pharmacokinetic parameters.
The occurrence and prevention of the M74 syndrome, a thiamine deficiency disease in Baltic salmon

The two most important pharmacokinetic parameters are the clearance and the volume of distribution of a drug. Clearance (CL) is the volume of blood that is completely cleared of the drug in a unit time, usually litres per hour or millilitres per minute. Total body clearance is the sum of all the different clearance processes occurring for a given drug, e.g. elimination in the urine and in the eggs and the metabolism of the drug. The volume of distribution relates the concentration of a drug in the blood to the total amount in the body. For example, if a drug has a blood concentration of 10 mg l⁻¹ when there is 1 g of drug in the body, the apparent volume of distribution (Vₐ) would be 100 l. Although a measure of the general tissue penetration, Vₐ does not, however, tell in which tissues the drug is distributed. Computer programmes can be used in the calculation of the pharmacokinetic parameters, but they can also be determined graphically from the time concentration curves. From Figure 7 for example:

\[
\text{Clearance CL} = \frac{\text{dose}}{\text{AUC}}
\]

\[
\text{Volume of distribution } V_{d} = \frac{\text{dose}}{\text{blood concentration at } c_{0}}
\]

The half life is the time needed for the blood concentration of the drug to fall by half. It can be determined separately for the distribution and elimination phases of the drug (Figure 7).

On the basis of the preceding subsection it is evident that the pharmacokinetic parameters are temperature dependent in fish. Usually, CL and Vₐ for example, are given for a certain water temperature, and withdrawal times of the drug-treated fish for human consumption are in day-degrees.

Figure 7. Time-concentration curve of a drug after a) intravenous and b) intraperitoneal injection. The area under the concentration curve (AUC) can be determined graphically (slashed area) or after modelling of the concentration. Drawing based on Figure 3-8 of Attila and Sandholm (1998), with permission of the copyright owner.
3. AIMS OF THE STUDY

The aims of the present work were to:

1. Describe and compare the occurrence of M74 syndrome in the Bothnian Bay rivers Kemi, Simo and Oulu, Daugava River in Latvia and the Atlantic salmon River Teno. Why do the stocks of some rivers suffer from M74 syndrome, while the disease is not found in others?

2. Compare the pharmacokinetics of thiamine hydrochloride in feral and farmed Baltic salmon and the distribution of thiamine in their tissues.

3. Examine the prevention of M74 syndrome with thiamine hydrochloride.
4. MATERIALS AND METHODS

4.1. Fish

4.1.1. Capture of feral and wild broodfish (I-IV)

The salmon for these studies were caught from the localities indicated in Figure 1 (I). The first broodfish were caught in the summer of 1993 (II) and the last in 2001 (IV). The fish from the mouth of Kemi, Oulu and Daugava Rivers were known to be feral, because there was no longer a spawning population in these rivers, and in the River Teno the collected fish were wild (the material was gathered in connection with the living gene bank project of the Finnish Game and Fisheries Research Institute). The River Simo still has a naturally spawning population of Baltic salmon, but it was also stocked with reared smolts and parr before the study years (Jokikokko and Jutila, 1998). The broodfish obtained from it and the main basin of the Baltic Sea were thus probably both feral and wild.

4.1.2. Maintenance of the fish at the fish farms (I-IV)

Broodfish

Most experiments and sampling for the thiamine measurements presumed that the caught fish were maintained at the fish farms or pens in the river (Teno and Daugava rivers), usually for weeks before the stripping or the beginning of the experiment. Only the fish caught from the Baltic main basin had contents in their alimentary canal during the sampling. There was no attempt to feed the feral or wild fish and the farmed females had been without food for 10-14 days before the experiments or sampling of their tissue material. During the experiments (III-IV) the fish were kept unfed.

At the farms the fish were kept in ordinary broodfish tanks in natural water temperatures. Most of the fish material was kept at the Lautiosaari fish farm of the Finnish Game and Fisheries Research Institute, where during their first week in captivity the broodfish were marked with a Carlin tag and given a single intramuscular injection of ampicillin trihydrate (15 mg kg\(^{-1}\), Penbritin vet. 150 mg/ml \(^{TM}\), Orion-Farmos, Finland or Duphacillin 150 mg kg\(^{-1}\) vet\(^{TM}\), Fort Dodge/Scanvet, Finland) to prevent clinical furunculosis. If needed the fish were bathed up to three times a week with formaline to prevent saprolegniosis. Neutral buffered tricaine methane sulphonate (MS-222\(^{TM}\)) was used for the anaesthesia, when ailing measures were performed for the fish.

In IV the broodfish were kept in flowthrough receptacles corresponding to those described in Soivio et al. (1975), but larger.

Eggs

The eggs were stripped when the females were ripe (when the eggs flowed easily). They were fertilised and the water-hardened eggs disinfected with an iodophor (100 ppm free iodine for 10-15 minutes). The ova were incubated in the hatchery water of their respective fish farms. All fish farms used local surface water, except that used in the incubation of the Teno River salmon, where untreated bore-hole water was used. Standard malachite green baths to prevent saprolegniosis of the eggs were used at the farm incubating the Daugava River salmon, but not at other farms incubating the egg material of this thesis. At these farms the dead eggs were removed soon after fertilisation and during the eyed-egg stage, when needed.
Yolk-sac fry

During the early yolk-sac period 100-200 healthy yolk-sac fry were put in 9 x 9 cm\(^2\) plastic or 9 x 18 cm\(^2\) aluminium trays to follow the mortality of the fry. At the farm incubating the eggs from Teno river the whole progeny of the females was followed in a similar way. The fry were observed daily and dead individuals were counted and removed every 1-3 days.

4.1.3. Biometrics and haematocrit (I, III-IV)

The total length (to the nearest cm) and weight (at least to the nearest one hundred grammes) was used in calculating the condition factor of the female brood fish according to the formula:

\[
\text{Condition factor (K)} = \frac{100 \times \text{total body weight (in grams)}}{\text{total body length}^3 \text{ (in cms)}}.
\]

For the calculation of the hepatosomatic (HSI) and gonadosomatic (GSI) indices of the females, the weight of the liver without the gallbladder and the weight of the eggs and the ovarian walls were measured. These indices were calculated according to the following formulae (the total body weight includes the weight of the liver and the gonads):

- Hepatosomatic index (HSI) = \(\frac{100 \times \text{liver weight}}{\text{total body weight}}\).
- Gonadosomatic index (GSI) = \(\frac{100 \times \text{gonad weight}}{\text{total body weight}}\), (Wootton, 1991).

The haematocrit values were calculated as a mean of the results of two measurements performed from the blood removed directly into the heparinised haematocrit capillaries from the 2 ml plastic syringe and injection needle used for the sampling. The capillaries were sealed with wax and centrifugation (Clay Adams Readacrit Centrifuge, Becton, Dickinson & Co., N.J., the U.S.A.) begun within 5 minutes from the sampling.

4.1.4. Mortality data of the yolk-sac fry and pathological and microbiological studies (I-III)

The yolk-sac fry in the experiments were examined daily and dead individuals counted. In III, samples of fry in their agonies were taken for thiamine analysis from the experiment. These were included in the mortality for the date in question. Cumulative mortalities were calculated until the end of the yolk-sac stage (when the thiamine treated siblings of the test fry had begun to eat artificial feed in I and III).

In order to exclude aetiologies other than M74 syndrome as a cause of the disease and mortality in the yolk-sac fry, normal diagnostic studies of a fish disease laboratory (macroscopic and histological pathology, parasitological examination for gill and skin parasites, virology and bacteriology) were carried out. These were performed according to Midtlyng et al. (1992) with small modifications described in more detail in III.

4.2. Treatments and experiments with the fish

Permits for the experiments were granted by the Provincial Office of Oulu.

4.2.1. Bathing of the yolk-sac fry with thiamine hydrochloride (III)

In Expt 1, 150-200 yolk-sac fry from each of seven females were bathed in 500 mg l\(^{-1}\), 2500 mg l\(^{-1}\) or 12500 mg l\(^{-1}\) thiamine hydrochloride for one hour. Thiamine hydrochloride solutions were made immediately before the bathing by dissolving thiamine hydrochloride Ph. Eur. (Oriola, Finland) in hatchery water. The pH of the solution was adjusted to that of the hatchery water (pH = 6.2-6.4) with sodium hydroxide p.a. (Merck, Germany). The water was provided with supplementary oxygenation during the treatment. A fourth series of fry, sham treated with hatchery water, served as controls. Thus the experiment comprised a total of 28 groups of yolk-sac fry.
A similar bathing, but with a thiamine hydrochloride concentration of 1000 mg l\(^{-1}\) for 1 hour, was given to a proportion of yolk-sac fry of 13 females in Expt 3, while the rest of these fry were left as untreated controls. Eight of the females had received a thiamine hydrochloride injection and 5 females left without.

The fry were observed daily and the dead individuals counted and removed every 1-3 days. A sample of yolk-sac fry from each of the females was taken for thiamine analysis 1-2 days before the bathing and the same was done later for fish found lying on their side on the bottom of the tray. The test fry in Expt 1 were not fed in the trays, but their siblings, which were being used for normal production of the farm (and were treated with 1000 mg l\(^{-1}\) thiamine hydrochloride for 1 hour), had begun to eat artificial feed at the same time as this experiment ended.

**4.2.2. Thiamine hydrochloride and astaxanthine injections of the female broodfish in the prevention of M74 syndrome (III-IV)**

The broodfish were anaesthetised and injected i.p. with thiamine hydrochloride and astaxanthine solutions, which were prepared to achieve a needed concentration of the substance to be injected at a dose of ca. 1 ml solution per 1 kg fish. The solutions were kept in melting ice in a covered styrofoam box and used not later than 3 hours after the preparation.

The thiamine hydrochloride solution was made by dissolving crystalline thiamine hydrochloride Ph. Eur. (Oriola, Finland) in distilled water. The pH of the solution was adjusted to 6.9-7.2 (IV) or 7-8 (III) with sodium hydroxide. Several doses of thiamine hydrochloride were used: 125 mg kg\(^{-1}\) (Expt 2 in III), 31 mg kg\(^{-1}\), 63 mg kg\(^{-1}\) and 131 mg kg\(^{-1}\) (IV). Crystalline astaxanthine (Roche, Finland) was suspended in a 0.5% solution of methyl cellulose Ph. Eur. 1500 (Tamro, Finland) in distilled water to obtain an astaxanthine concentration of 12.5 mg l\(^{-1}\). The dose of the astaxanthine was 11 mg kg\(^{-1}\).

In Expt 2 of III, four treatment groups of the feral Baltic salmon broodfish females were established:
1. Thiamine group, 125 mg kg\(^{-1}\), 19 females,
2. Astaxanthine group, 11 mg kg\(^{-1}\), 10 females
3. Thiamine + astaxanthine group, 125 mg kg\(^{-1}\)+11 mg kg\(^{-1}\), 10 females
4. Control group, no treatment, 9 females

The injections were performed ca. 1400-1640 day-degrees before the stripping of the females and the end of the experiment.

In IV farmed females were i.p. injected in Expt 1 as described in subsection 4.2.3 with a dose of 132 mg kg\(^{-1}\).

In Expt 3 of IV, four treatment groups of the feral Baltic salmon broodfish females were established: 9 females were injected i.p. with a dose of 31 mg kg\(^{-1}\), 8 with a dose of 63 mg kg\(^{-1}\), 10 with a dose of 131 mg kg\(^{-1}\) and 7 left untreated controls. The injections were given ca. 1450-1540 day-degrees before the stripping of the females and the end of the experiment.

**4.2.3. Trials on pharmacokinetics (IV)**

Intra-aortic (i.a.) and i.p. injections were performed in Expt 1 after a permanent cannulation of the dorsal aorta according to Soivio et al. (1975). There were small modifications to the original method in the flushing back of the blood in the cannula with the heparin solution. These are presented in IV. The blood sampling was performed from conscious fish through the aortic cannula or from the caudal vein of the lightly sedated fish.

Expt 1 was performed to estimate the relative bioavailability of the i.p. injected thiamine hydrochloride with reference to the i.a. route of administration and to ensure that the i.p. route was appropriate in the second experiment. Ten samplings after the injection a 66 mg kg\(^{-1}\) median dose
appropriate in the second experiment. Ten samplings after the injection a 66 mg kg\(^{-1}\) median dose of neutral buffered thiamine hydrochloride in the i.a. and 132 mg kg\(^{-1}\) in the i.p. administration were performed. The first sampling was done 0.5 hour and the last 25 days after the injection.

In Expt 2 the pharmacokinetics of thiamine hydrochloride was studied after i.p. administration as described in the previous subsection. Blood samples were taken from the caudal vein of a lightly sedated fish, the first 1 hour and the last 45 days after the injection.

In Expt 3 the effect of different doses of i.p. injected thiamine hydrochloride on the concentrations of different forms of thiamine and TTH in the newly stripped eggs was studied in feral Baltic salmon females (see subsection 4.2.2).

4.2.4. Field trials with wild salmon (III)

In the field trial in the River Simo in 1996-1997 and Kiiminki river in 1997, all Baltic salmon broodfish on their spawning migration up the river were attempted to be caught with a trap net. Of these, 215 fish in the River Simo (Erkki Jokikokko pers. com. 2004) and 8 fish in the River Kiiminki (Pekka Hyvärinen pers. com. 2004) were injected with thiamine hydrochloride at a dose of 1 ml kg\(^{-1}\) fish i.p. with a solution prepared at the river bank immediately before use: 2 g NaHCO\(_3\) (sodium bicarbonate) was mixed with 40 ml of sterilised water. The liquid was gradually added to a tube containing 5 g crystalline thiamine hydrochloride Ph. Eur. (Oriola Finland). The pH of the solution was thus adjusted to ca. 6.8 and a ca. 115 mg ml\(^{-1}\) thiamine hydrochloride solution was achieved. Neutral buffered tricaine methane sulphonate (MS-222™) was used for light anaesthesia and the thiamine hydrochloride injection with a dose 115 mg kg\(^{-1}\) given in front of the pelvic fins (Figure 8). The fish was allowed to recover in fresh water and released to continue its migration.
4.3. Chemical methods

All samples were analysed in duplicate. The results of the thiamine and astaxanthine analysis are given as the mean on a wet weight basis. The reported concentrations have not been adjusted for percentage recoveries. The EROD activities are given in pmol mg\(^{-1}\) protein min\(^{-1}\).

4.3.1. Sample handling (I, III-IV)

The blood, liver and muscle samples were taken within 15 minutes of stunning. The egg and tissue samples were enclosed in polypropene tubes, frozen in liquid nitrogen (not later than 15 minutes after sampling) and kept at -70 °C to await chemical analysis. In I at location F the samples were, for practical reasons, kept on melting ice for 2-8 hours before being frozen in liquid nitrogen.

In the EROD analysis (Expt 3 in III) the liver of 20 decapitated yolk-sac fry (two pools with 10 livers in each) from each of the groups were sampled into small polypropene tubes in melting ice. The livers were frozen in liquid nitrogen within 10 minutes of decapitation of the fry and kept at -70 °C until the analysis of EROD activity.

4.3.2. Thiamine analysis (I, III-IV)

Total thiamine (I, III-IV)

Total thiamine concentration was analyzed by high performance liquid chromatography (HPLC) (Ollilainen et al., 1993), with enzymatic hydrolysis modified according to Hägg (1995). The sample was autoclaved in hydrochloric acid and hydrolysed overnight with claradiastase enzyme. The thiamine was oxidised to thiochrome with potassium ferricyanide. After purification the thiochrome was analysed by HPLC. The details of the sample preparation, apparatus, operating conditions and reagents are given in III and Löflund et al. (1999), where the procedure has the code LAB 2. The detection limit for total thiamine in this system was estimated to be 15 ng g\(^{-1}\) tissue. Intratest reproducibility (variation within one day), calculated as a coefficient of variation (CV%), was 4.0% for the egg samples and 4.6% for the liver samples, and recovery percentages were 61-87% for the liver samples and 72-100% for the egg and fry samples.

Assay for thiamine and its phosphates (IV)

The thiamine and its phosphate esters were analysed with a slight modification of the method of Brown et al. (1998). The HPLC instrumentation consisted of two Waters 510 HPLC pumps, a Waters 717 plus autosampler connected to a sample tray cooler operating at 4° C and a Waters 474 fluorescence detector. Quantification was performed with Millenium 32 ver 4. chromatography workstation. The column was a polymer based Hamilton PRP-1 (150 x 4.1 mm) thermostated to 40 °C. Thiochrome detection was performed at an emission wavelength of 435 nm and an excitation wavelength of 366 nm. The details of the analysis are given in IV.

The detection limits for the different forms of thiamine in whole blood using the method in Expt 2 were as follows: TH 0.6 ng g\(^{-1}\), TMP 1 ng g\(^{-1}\) and TPP 2 ng g\(^{-1}\). The concentration of TTH was calculated as the sum of the three forms. Coefficients of variation for the three compounds were 7.8%, 4% and 13%, respectively, while the recovery percentages were 119%, 90% and 102%. The respective detection limits were 2.3 ng g\(^{-1}\) for TH and TMP and 3 ng g\(^{-1}\) for TPP in the eggs, 0.6, 2 and 5.5 ng g\(^{-1}\) in the muscle and 1 ng g\(^{-1}\) for both TH and TMP and 2 ng g\(^{-1}\) for TPP in the liver.
4.3.3. Astaxanthine analysis (III)

Astaxanthine was analyzed by a modification of the HPLC method presented by Christophersen et al. (1989). The homogenised tissue was mixed with anhydrous sodium sulphate and the mixture was allowed to stand for 1 hour. The colour of the sample was extracted with acetone, evaporated and dissolved in ethanol (eggs and yolk-sac fry) or hexane (muscle). The uncoloured lipids were removed, after which the evaporation was repeated and the pigments dissolved in ethanol. The astaxanthine was measured by HPLC using a UV-detector. The details of the sample preparation, apparatus, operating conditions and reagents are given in III.

4.3.4. EROD analysis (III)

A gradient centrifugation in saccharose was performed for homogenised liver tissue. The EROD activity was measured from the post-mitochondrial S-9 fraction with the fluorescence method described by Hodson et al. (1991), with the exception that an incubation temperature of 20 °C was used. The details of the EROD analysis are given in III.

4.4. Pharmacokinetic and statistical methods

4.4.1. Pharmacokinetic analysis (IV)

The concentrations of the different forms of thiamine and TTH in the blood were studied by plotting the common logarithm of the median concentration against the time after the thiamine hydrochloride administration. Before calculation of the pharmacokinetic parameters, all measured blood thiamine concentrations were baseline corrected by the concentrations measured prior the dosing of thiamine hydrochloride. The time when the median blood thiamine concentrations had decreased to the baseline levels was estimated by graphical extrapolation (Fig. 1 in IV). These values were used in addition to the measured concentrations in the calculation of the areas under the whole blood concentration time curve (AUC) for the i.p. and i.a. injected fish. The half-lives of the distribution ($t_{1/2\alpha}$) and elimination phases ($t_{1/2\beta}$) were also estimated graphically on the basis of Figure 1 in IV.

The calculations of the relative bioavailability (F) of i.p. and i.a. injected thiamine hydrochloride were based on equation 5.4 in Birkett (1998):

$$F\, (\%) = (\text{Dose}_{\text{i.a.}} \times \text{AUC}_{\text{i.p.}} / \text{Dose}_{\text{i.p.}} \times \text{AUC}_{\text{i.a.}}) \times 100,$$

where AUC = area under the whole blood concentration time curve and the subscripts i.a. and i.p. refer to the routes of injection.

4.4.2. Statistics (I-IV)

In data where the parameter is asymmetric, as in the cumulative mortality for M74 syndrome, it is inevitable that nonparametric statistics must be used. The significance of the differences in the thiamine, astaxanthine and mortality data between the treatment or sampled fish groups was usually studied using the Kruskal-Wallis test (I, III-IV). The Mann-Whitney U-test after the Bonferroni method (Sokal and Rohlf, 1995) was then performed to decide which populations were not identical to the others. When the groups were not independent (II-III), Wilcoxon’s signed rank test was used for the comparison of two groups. In repeated measurement of the mortality data (III) the Friedman test ($\chi^2$ statistic) was used. After this the sign test with the Bonferroni adjustment (Sokal and Rohlf, 1995) was used to determine which treatment results were not identical to the others.
The associations of the parameters were tested mainly by the Spearman rank correlation test (I, III-IV). The G-test was used to study the effect of the injections on the mortality data of the injected broodfish (III). The reciprocal and logarithmic transformations of the original data were used to fulfil the requirements of regression analysis in IV.

The statistical software packages SPSS/PC+ (I, Norusis, 1986) and Statistics for Windows (I-IV, Analytical Software, 1996 and 2000) were used in carrying out the statistical analysis. Diem and Lentner (1971); Shott (1990) and Sokal and Rohlf (1995) were consulted in choosing the appropriate test and obtaining p-values.
5. RESULTS

5.1. Occurrence of M74 syndrome in the salmon of the Bothnian Bay rivers of Finland, Daugava River and the Atlantic River Teno (I)

All the observations of the mortality of yolk-sac fry were made at fish farms in 1995-1997. They represent follow-up of the fry of stripped wild or feral broodfish and not the naturally spawning fish in the river. The median cumulative mortality of the yolk-sac fry from the early yolk-sac period to the swim-up was only 1% (range 0-16%) in the River Teno salmon, 9% (1-53%) in the River Daugava and 100% (7-100%) in the Bothnian Bay Rivers Kemi and Simo. The Bonferroni-adjusted Mann-Whitney test confirmed that mortality was statistically significantly lower in the Daugava River than in the Rivers Simo (p<0.01) and Kemi (p<0.05), while mortality in the River Teno was significantly lower than at all other sites (p<0.01) (I, Table 3). Symptoms of M74 syndrome were only seen in the fry originating from the feral females of the Bothnian Bay rivers (I-III). Not even the offspring with the highest observed mortality level from the Daugava River data (53%) showed symptoms of M74 syndrome. TTH concentration in the egg batch from which these fry hatched was 2.2 µg g⁻¹, well above the critical value found for the M74 syndrome in I.

Newly-stripped eggs with TTH concentrations of ca. 0.35 µg g⁻¹ or lower were shown to develop into M74 offspring (I). Not a single female of the Rivers Teno and Daugava produced eggs with such low TTH concentrations, while the majority of the tested females in the Bothnian Bay rivers did have such low concentrations in their newly stripped eggs [median (range) for the Rivers Kemi 0.3 (0.2-0.4 µg g⁻¹), Simo 0.3 (0.2-0.8 µg g⁻¹) and Oulu 0.5 (0.2-1.7 µg g⁻¹)] (I, Table 3).

5.2. Parental background as a predisposing factor for M74 syndrome in the Baltic salmon fry (II)

In a cross-fertilisation experiment the eggs of 8 farmed and 8 feral or wild females were fertilised with the milt of 8 farmed and 8 wild male Baltic salmon. The milt of each individual male fish was used to fertilise eggs from one farmed and one feral or wild female. Almost all yolk-sac fry derived from wild females died. The median (range) cumulative mortality from hatching to the exhaustion of the yolk-sac fry was 97% (48-100%). In the farmed females this was 2% (0-24%). Besides this marked maternal effect there was also a minor effect of male background on the level of the mortality of fry suffering from M74 syndrome: Crossings of wild females with wild males resulted in greater mortalities (median 99%, range 92-100%) than with farmed males (97%, 48-100% respectively). According to the Wilcoxon signed rank test these differences in the yolk-sac fry mortalities are significant (p < 0.001 for female, p < 0.05 for male effect).

5.3. Thiamine concentrations and metabolism

5.3.1. Yolk-sac fry (I, III)

The TTH concentrations in the yolk-sac fry are presented in Figure 9. The median (range) TTH concentrations of the yolk-sac fry, which soon suffered from M74 mortality (at least 90% cumulative mortality), was 0.19 (0.06-0.23 µg g⁻¹, N = 18) in the material from the Rivers Kemi and Simo in 1994-96 (I) and 0.16 (0.10-0.17 µg g⁻¹, N = 7) in the material from the River Simo in 1994 (I). The median and range of the concentrations were, however, not presented in the original article. The bathing of the yolk-sac fry in 1000 mg l⁻¹ thiamine hydrochloride for 1 hour before symptoms of typical M74 (100% mortality in a few days) were evident in the fry (350 day-degrees) was shown to elevate the median TTH concentrations from 0.23 µg g⁻¹ to 2.20 µg g⁻¹ (515 day-
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degrees) and to prevent the symptoms of M74 (III, group D of Table 3, N = 5, Wilcoxon matched-pairs signed rank test, p < 0.05). The effect of the preventive thiamine hydrochloride bathing on the mortality in M74 syndrome was shown to be dose dependent (III, Fig. 2 a).

Intraperitoneal injections of thiamine hydrochloride in the female (ca. 100 mg kg⁻¹ fish) also resulted in the elevation of the TTH concentrations in the fry (median concentrations in the noninjected group 0.22 µg g⁻¹, N = 8 and 3.95 µg g⁻¹, N = 11 in the injected ones, p < 0.001, Mann-Whitney test after Bonferroni adjustment, III, Table 2) and in the prevention of M74 syndrome. The injections of astaxanthine in the females, although having no preventive effect on the mortality in M74 syndrome, perhaps had an enhancing effect on the use of the thiamine during the embryonal or early yolk-sac stage of the offspring (III).

Figure 9. Total thiamine concentrations (µg g⁻¹) in the newly stripped eggs and yolk-sac fry prior to symptoms of M74 in the offspring originating from River Simo and Kemi broodfish caught in 1994-96. Each square represents a sample of the offspring of one female brood fish. N*: number of family groups examined. Criteria for the categories: eggs/yolk-sac fry developing into M74 offspring: cumulative yolk-sac mortality 90-100% developing in a few days; eggs/yolk-sac fry developing into offspring with cumulative yolk-sac fry mortality of 20-90%, eggs/yolk-sac fry developing into normal offspring: cumulative yolk-sac fry mortality under 20%.
5.3.2. Eggs (I)

The correlation of the thiamine concentrations at the yolk-sac fry and in the newly stripped eggs was evident (I, Fig.3). The occurrence of “classical” M74 syndrome with nearly total mortality of the fry group within a few days was shown to occur only in the offspring developing from egg batches having TTH concentrations under 0.37 µg g\(^{-1}\) (N = 22), and none of those showing lower grade mortality had concentrations under 0.34 µg g\(^{-1}\) (N = 15). The critical TTH concentrations in the newly stripped eggs were thus clearly defined and there was less overlapping than in the concentrations in the yolk-sac fry (Figure 6). The thiamine supply of the female broodfish was manipulated by injections and diet. The effect of these on the thiamine concentrations in the eggs is summarised in subsection 5.3.3.2.

5.3.3. Female broodfish (I, III-IV)

Thiamine in the tissues of salmon of different origin and background (I, III)

The concentrations of TTH in the newly stripped eggs of the Bothnian Bay rivers of Finland were low, as presented in subsection 5.1. The median concentrations in the white muscle of the Baltic salmon females were only 0.5 µg g\(^{-1}\) (fish caught from the Baltic proper and from the mouth of River Simo) – 0.8 µg g\(^{-1}\) (fish caught the mouth of Daugava River), while the respective value for River Teno salmon was 2.1 µg g\(^{-1}\) (p < 0.01, Mann-Whitney tests after Bonferroni adjustment, I, Table 3). There was a correlation in the Baltic salmon material between the TTH concentrations in the white muscle and newly stripped eggs, but not in the material of the River Teno as a whole. This is illustrated in Figure 10.

![Figure 10](image-url)

Figure 10. Scatterplot between the TTH concentrations in the white muscle and eggs of female broodfish. There is a significant positive linear correlation in the materials of the River Simo Suluissa oleva lause pitäisi olla: (Spearman rank correlation test, \(r_s\)=0.6611, p < 0.05, N = 13) and Daugava River (\(r_s\)=0.8511, p < 0.01, N = 10), but not in the River Teno.
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In the liver of the females there was a corresponding tendency of the fish caught from the mouth of the Bothnian Bay rivers to have the lowest median concentrations (1.6-2.0 µg g⁻¹, N = 28). The Daugava, Teno and Baltic proper salmon had higher medians (2.8-3.7 µg g⁻¹, N = 42), which did not differ significantly from each other. The highest median was found in the Baltic proper females, which were still feeding (I, Table 3). There was correlation between the TTH concentrations in the liver and eggs of the Daugava River females only, but not in the material of the Bothnian Bay and Teno rivers (I, Figure 5 b).

The TTH concentrations were roughly double in the blood, liver and newly stripped eggs of farmed females that had received a diet supplemented with thiamine and astaxanthine as compared with those on an unsupplemented diet (N = 32, p < 0.001, Mann-Whitney test results, III, Table 2).

5.3.3.1. Certain aspects of pharmacokinetics of thiamine in the Baltic salmon (IV)

The median concentrations of TTH in the blood of farmed Baltic salmon females already reached their peak values in the samples taken 9 hours (ca. 2 day-degrees) after the i.p. injection of thiamine hydrochloride (132 mg kg⁻¹ fish, IV, Fig. 1). After this, there appeared to be a linear distribution phase, which changed to a linear elimination phase with a much longer half-life at ca. 20 day-degrees after the injections, both by the i.p. and i.a. injection route. The relative bioavailability of i.p. injected thiamine hydrochloride was shown to be ca. 94% against an i.a. reference dose.

In a 489 day-degree experiment on the kinetics of i.p. injected thiamine hydrochloride, great differences in the incorporation of thiamine in blood, liver, white muscle and newly stripped eggs were found between female broodfish with feral and farmed backgrounds. The whole blood TTH concentrations rose higher in the feral fish and maintained their higher values until the end of the experiment (IV, Table 3). The median TTH concentrations in the liver at the end of the experiment were also higher in the feral females. The median concentrations in the skeletal white muscle and newly stripped eggs of the farmed females were, however, more than double those in the feral fish (IV, Fig. 3). The relative proportions of injected thiamine hydrochloride found in the different tissues at the end of the experiment in the feral fish were as follows: eggs 2.4%, muscle 7.2%, liver 0.1 and in the blood during the peak concentration 11.2%. The corresponding proportions in the farmed females were 7.8%, 18.5%, 0.2% and 8.5%.

The absorption of TH into the blood was almost entirely behind the elevation of the whole blood TTH concentrations. The tissues differed markedly in the distribution of thiamine into the different forms: In the blood and liver 80-90% and in the eggs even more than 90% of the TTH was TH, but in the muscle the reverse situation was observed and over 90% occurred in phosphorylated forms, mainly TPP. In the uninjected female broodfish, however, the distribution of the different forms of thiamine in the eggs resembled that of the muscle tissue in the thiamine hydrochloride treated females (IV, Fig. 4).

The i.p. injection of astaxanthine (ca. 11 mg kg⁻¹ fish) had no effect on the TTH concentrations in the newly stripped eggs of female broodfish, either in females that received only astaxanthine or in those that also received an i.p. injection of thiamine hydrochloride (ca. 100 mg kg⁻¹ fish, I, Table 2).
5.4. Use of thiamine hydrochloride in the prevention of M74 syndrome (III-IV)

5.4.1. Bathing of the yolk-sac fry (III)

The bathing of the 282-383 day-degrees old (from fertilisation) fry showing no symptoms of M74 syndrome with a thiamine hydrochloride concentration of 500 mg l\(^{-1}\) for 1 hour delayed the onset of the disease by ca. 50-100 day-degrees. Bathing with concentrations of 2500 mg l\(^{-1}\) and 12500 mg l\(^{-1}\) for 1 hour had a ca. 300 day-degree effect (III, Fig. 2 a). At the Lautiosaari Fish Hatchery, bathing in 1000 mg l\(^{-1}\) for 1 hour has been used in the prevention of M74 syndrome, given at an age of ca. 300 day-degrees after fertilisation. Some of the fry need to be re-treated in the late yolk-sac period (III), sometimes even 2-3 times (J. Rytilahti pers. comm.).

The bathing in the concentrations of 500-12500 mg l\(^{-1}\) for 1 hour had no observable effect on the behaviour or on the mortality of the fry during the first 2 days after the bathing (III). No negative effects of the thiamine hydrochloride treatments on the growing parr or smolt have been observed at the fish farms of northern Finland. This is based on the diagnostics of fish diseases in the area carried out at the National Veterinary and Food Research Institute, Oulu, during a 10–year period when thiamine hydrochloride bathings have been performed.

5.4.2. Injection of the female broodfish (III-IV)

Feral Baltic salmon female broodfish were i.p. injected with thiamine hydrochloride after they had been caught from the river in June-July (ca. 1500 day-degrees before the spawning time). This resulted in elevated concentrations of TTH in the newly stripped eggs in a dose dependent manner according to the formula (R\(^2\) = 0.872, p < 0.001):

\[
\log(TTH) = 0.927 - 23.215 \times [1/(x+30)],
\]

where \(x\) = the dose of thiamine hydrochloride in mg kg\(^{-1}\) fish.

On the basis of Fig.5 in IV it was estimated that with 95% probability 2-4.7 µg g\(^{-1}\) TTH concentration in the newly stripped eggs was achieved with an i.p. thiamine hydrochloride dose of ca. 19 mg kg\(^{-1}\).

The i.p. injection of thiamine hydrochloride resulted - in addition to the prevention of the M74 syndrome – also in a greater “GSI” (proportion of the weight of the stripped eggs among the total body weight of the female): In the control females the median (range) “GSI” was 16.3% (15.4-17.9%) and in the fish having received the thiamine hydrochloride injection it was 17.7% (15.4-20.3%), p<0.01, Mann-Whitney test.

The preventive i.p. injections of thiamine hydrochloride (or astaxanthine) did not affect the mortality of the feral females compared to the untreated ones (III). In the field trial of the treatment of wild and feral broodfish of Baltic salmon migrating up the Simo river in 1996, only 1 female of the injected 63 females was found dead from the river after release (Jokikokko and Pylväs M, (1997), unpublished statistics of the National Veterinary and Food Research Institute, Oulu).
6. DISCUSSION

6.1. Occurrence of M74 syndrome in the salmon of the Bothnian Bay rivers of Finland, Daugava River and the Atlantic salmon River Teno

M74 syndrome was shown to be common in the offspring of the female feral or wild broodfish of the Bothnian Bay rivers of Finland in the mid-1990s (I-III). The present results (I) also experimentally confirm the information of the report of Mitans (1994) and the unpublished information of Inari Aquaculture, Inari, Finland, that M74 syndrome does not exist in the offspring of the feral and wild salmon broodfish of the Baltic River Daugava or the Barents Sea River Teno.

The mortality figures obtained from the normal production of fish farms keeping fry with M74 syndrome may be somewhat exaggerated. The dead or dying fry must be removed every day to prevent the growth of the Saprolegniaceae in the fry. The fungal growth in the masses of the dead fry may otherwise also lethally infect some of the healthy fry on the hatching tray. It is, however, questionable whether the trays can be continuously kept clean enough during the normal production routine on a fish farm. Our figure of a 71% mean yolk-sac mortality in the offspring of 13 females of the River Simo strain (I), however, corresponds well with that reported by the Finnish Game and Fisheries Research Institute (Figure 2) for that year.

The differences in the occurrence of M74 syndrome in the different stocks of Baltic salmon are most probably linked to the diet of the female during the feeding migration. The absence of M74 syndrome in the River Daugava stock might be a consequence of the later return and continuation of eating closer to the onset of spawning, as was proposed in (I) and by Hansson et al. (2001). One can also question whether the rapid rate of egg and yolk-sac development (low number of day-degrees needed for a specific stage of development, chapter 2.4.1. in this thesis) somehow predisposes the northern Baltic salmon stocks to thiamine deficiency. The diet of wild Atlantic salmon in the feeding areas of the stocks of northern latitudes is very different from that of the Baltic salmon (Karlsson et al. (1999), Hanson et al. (2001), Jacobsen and Hansen (2001)). This is the obvious reason for the absence of M74 syndrome in the River Teno salmon. The exact nature of the different diet related factors in the pathogenesis of M74 syndrome reviewed in chapter 2.2.3. would, however, require much more research. Especially the interactions of the nutrition of salmon, toxicological factors and the Baltic Sea food web of salmon should be studied.

There have been no direct observations of M74 mortality in natural rivers. If the offspring of a female died on the bed of the spawning river, the nearby normal fry or parr would be able to compensate for the effect of M74 mortality on a certain stretch of a river. The annual figures obtained from the offspring of the females stripped at fish farms cannot be used to provide a direct measurement of the effects M74 syndrome in natural rivers. Such effects on the natural production of the Bothnian Bay rivers have, however, been reported on the basis of electrofishing surveys: Karlström (1999) found a 63% reduction in the number of parr produced per ascending spawner in the River Tornio when he compared the result of the spawn during the severe M74 syndrome years from 1992-1996 (mean mortality 61%, range 52-74%) to those of lower years, 1988-1991 and 1997 (mean 15%, range 7-33%). Jokikokko et al. (1995) reported corresponding data from the River Simo. It is probable that M74 syndrome has an effect on the natural salmon population, especially when the prevalence of the syndrome is high and it occurs in the rapids where only a few females spawn. On the stock level of a large river the effects may not be seen. Romakkaniemi et al. (2003) suggested that the effects of M74 syndrome on the stock of River Tornio salmon were counteracted by fishery regulations and a good spawning stock, even in the worst M74 years in the 1990s.
6.2. Parental background as a predisposing factor for M74 syndrome in Baltic salmon fry

M74 syndrome has been known to occur in the offspring of certain females since its first observation in the Bergeforsen fish hatchery in 1974. The whole offspring of certain females was reported to die in 3-5 days after the first signs of the syndrome (Börjeson et al., 1994). According to the authors, milt from wiggling males gives healthy offspring. However, nearly all wiggling females produce offspring with M74 syndrome (Börjeson and Norrgren, 1997). These studies were based on observations at fish farms, but a male effect on the cumulative mortality was found in the cross-fertilisation experiment of II. The difference in the cumulative mortality between the offspring of the farmed and wild/feral males was small, but systematic. The male effect is difficult to explain, but cannot depend on the negligible thiamine contribution of the sperm cell to the zygote. The male can contribute enzymes that are important in thiamine metabolism, such as transketolase and glucose-6-phosphate dehydrogenase (Amcoff et al., 2002a). This role would, however, need more research. Even if there were sexually mediated genetic effects on these or in carotenoid metabolism (Lundström et al., 1999b; Pettersson and Lignell, 1999; III), further studies would be needed to identify possible differences between farmed and feral males.

6.3. Thiamine concentrations and metabolism

6.3.1. Yolk-sac fry

The TTH concentration ranges (0.06-0.23 µg g⁻¹, N = 18 and 0.10-0.17 µg g⁻¹, N = 7) in the fry having M74 syndrome in I and III are apparently slightly higher than the concentrations reported by Amcoff et al. (1998a and 1998b), where the mean (SD) of TTH was ca. 0.04 (0.03) µg g⁻¹ (N = 8), 0.06 (0.04) µg g⁻¹ (N = 4) and 0.08 (0.04) µg g⁻¹ (N = 6). The later values of Amcoff et al. (1999a) have been higher, with the means of three groups of different years and rivers being ca. 0.03-0.20 µg g⁻¹ (N = 26). Our results for yolk-sac fry correspond well with the higher mean values of Amcoff et al. (1999a) and the values reported for a thiamine deficiency syndrome (Cayuga Syndrome) in landlocked Atlantic salmon in New York’s Finger Lakes (Fisher et al. 1996). If the comparison is made on the basis of the threshold values proposed by Amcoff (2000) ca. 0.09-0.17 µg g⁻¹ and Fisher et al. (1996) ca. 0.12-0.23 µg g⁻¹, the present results, 0.16-0.23 µg g⁻¹ (I), again resemble more closely those presented for the Cayuga Syndrome. Although a difference certainly exists between laboratories in the measured concentrations (Löflund et al., 1999), there may be a real difference in the thiamine status of the yolk-sac fry of the salmon broodfish taken from the two Swedish and Finnish rivers of the Gulf of Bothnia. Wiggling behaviour in the broodfish was exceptional in our material, for instance, whereas it is not uncommon in Sweden (Börjeson et al., 1994). Amcoff et al. (1998b) reported much lower thiamine concentrations in the ovaries of wiggling than normally behaving females with offspring having M74 syndrome. The effect of the wiggling females is supported by the mean TTH concentrations in the normal yolk-sac fry in the Swedish material, 0.21 µg g⁻¹ (N = 6), 0.30 µg g⁻¹ (N =4) (Amcoff et al., 1998a) and 0.51 µg g⁻¹ (N = 8) (Amcoff et al., 1998b), being in the range of the TTH concentrations in I, 0.16-0.63 µg g⁻¹ (N = 6).

Effect of astaxanthine

The finding of the astaxanthine injected females giving rise to offspring that used their TTH reserves more efficiently than the control offspring of uninjected females supports the suggestion of Lundström et al. (1999b) that M74 syndrome might be linked to the oxidative stress of the yolk-sac fry. Amcoff et al. (2000) showed M74 affected yolk-sac fry to have greatly reduced activities of the thiamine dependent enzymes α-KGDH and transketolase in the liver. Gibson and Zhang (2002), in their review based on mammalian research, named these enzymes, especially α-KGDH,
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to be among the most sensitive enzymes to oxidative stress. Whether the astaxanthine injection
prevented the inactivation of thiamine dependent enzymes in the developing egg or fry of our study
(III) remains, however, an open question.

6.3.2. Eggs

The range in the concentrations of TTH in the newly stripped eggs of females producing fry with
M74 syndrome (0.18-0.37 \( \mu g \) g\(^{-1}\), N=22, I) is fairly similar to the mean ± 2*SD reported by Amcoff
et al. (1999a) for newly fertilised eggs in the Lule River on the Swedish side of Bothnian Bay,
which later developed M74 fry (0.18-0.48 \( \mu g \) g\(^{-1}\), N = 15). As was discussed in the previous
subsection, the wiggling females have lower TTH concentrations in their eggs.

The finding of a fairly sharp threshold value of TTH in the eggs (0.34-0.37 \( \mu g \) g\(^{-1}\)) and more
overlapping with the similar threshold value in the yolk-sac fry (0.16.-0.23 \( \mu g \) g\(^{-1}\)) in the prediction
of the M74 syndrome (I) is different from the reported corresponding concentrations from Sweden
(Amcoff et al., 1998b) and thiamine deficiency syndrome in landlocked Atlantic salmon in New
York (Fisher et al., 1996; 1998). The reasons for this are difficult to explain.

The thiamine concentration data in the eggs and fry have not been correlated in other studies, but
Figure 3 in I parallels the findings of Sato et al. (1987) of an approximately linear decrease in
thiamine levels in rainbow trout relative to degree-days from fertilisation to first feeding. On the
basis of Figure 3 in I and the high Spearman rank correlation coefficient (ca. 0.917), it is concluded
that the TTH concentration in the eggs of feral or wild salmon is a good predictor of the total
thiamine concentration in the yolk-sac fry before the appearance of M74. Fisher et al. (1998) also
reported regressions between the survival of the yolk-sac fry and the TTH concentrations in the
eggs and fry in the Cayuga syndrome of Atlantic salmon in the USA.

6.3.3. Female broodfish

6.3.3.1. Thiamine in the different tissues

The concentrations of TTH in the eggs of the salmon of different rivers were discussed in the
previous subsection. The association between the TTH concentrations in the eggs and liver has
received some research attention: Amcoff et al. (1996) found no correlation between TTH
concentrations in these tissues in their study of wiggling Baltic salmon females. Amcoff et al.
(2002b) used TTH concentrations in the liver as a measure of the thiamine status of the female
and found lower concentrations in the wiggling females than in the normally behaving fish, both
in the liver and in the eggs. In I a correlation between the TTH concentrations in the liver and eggs
was only found in Daugava River, i.e. in the location where fish did not suffer from M74 syndrome
or have very high TTH concentrations, as in the River Teno. In fish from the Bohman Bay rivers of
Finland no such correlation was found. This is in concordance with the result of Amcoff et al.
(1996). The liver has, however, only a limited capacity to store thiamine, as was shown in I. In the
skeletal muscle there is a much higher capacity for thiamine storage (I, IV) and it would probably
be more appropriate to use the concentrations in the skeletal muscle than in the liver for
measurement of the thiamine status of the salmon broodfish. Fisher et al. (1998) also showed a
regression between the maternal whole blood and egg TTH and TH concentrations in landlocked
Atlantic salmon that were free of Cayuga syndrome, but not in those suffering from it. Blood and
liver tissues probably reflect the need for thiamine in the developing eggs more rapidly than the
skeletal muscle. The female broodfish that were injected with thiamine hydrochloride in Expt 3 of
IV had higher “GSI” values (the proportion of the weight of the stripped eggs of the total weight)
than the control fish. This might indicate that the deficiency of thiamine has an effect on the egg
production in female Baltic salmon broodfish.
6.3.3.2. Aspects of the pharmacokinetics of thiamine in Baltic salmon

The relative bioavailability of the i.p. injected thiamine hydrochloride was shown to be nearly complete in Expt 1 (IV). The rapidity of the absorption phase and effective distribution could explain the high thiamine concentrations in eggs resulting from i.p. injection of thiamine hydrochloride after various time-intervals, which has been found in Börjeson et al. (1999) and III. In the distribution and elimination phases of Expt 1 (IV) the TTH concentration curves ran parallel, which together with the similar relative bioavailability of i.a. and i.p. injections make studies on the pharmacokinetics of thiamine using the latter administration route appropriate.

The concentrations of TTH in the blood rose sharply after the injection, which indicates good absorption from the abdominal cavity in both feral and farmed females in Expt 2 (IV). The times taken to reach the highest concentrations of TTH in blood were ca. half of those in Expt 1 (IV), which is in accordance with the ca. 10 ºC higher water temperature at the beginning of Expt 2 (IV). The similarity of the curve of TTH in blood, including the times of maximum concentrations in both feral and farmed females, suggested that the longer starvation of the feral fish did not cause major changes in the absorption of thiamine from the peritoneal cavity (starving is known to decrease the metabolic rate in salmonids, especially at the beginning (Smith, 1982)). The process of plasma TH transportation into and phosphorylation in the blood cells was evidently much slower than the absorption, which can be seen in the ca. 5 days needed to reach the maximum blood concentrations of TPP. Most TPP was probably located in the erythrocytes, but some may also have been present in the white blood cells and thrombocytes. Again, there was no evidence of a slower absorption or phosphorylation of thiamine into TPP in the feral females compared with the more recently fed farmed ones.

In Expt 2 (IV) a considerable proportion of the injected thiamine rapidly appeared in the blood. At the end of this experiment the tissues with a high hematocrit, namely the blood and liver (Bushnell et al., 1998), were found to have only a fraction of the TTH concentrations of the low grade perfusion and metabolically less active tissues such as white skeletal muscle (Bushnell et al., 1998) and eggs. Bird oocytes have thiamine binding proteins that are involved in the supply of thiamine to the developing embryo (White, 1987), but such have not been reported from fish. The high concentrations of TTH in the eggs and muscle indicate, however, some kind of intracellular thiamine “trapping” in these low perfusion tissues. The accumulation of TPP in the muscle is highly significant, because the highest concentrations of the phosphorylated forms (sum of TPP and TMP) found in the blood were so low that it would mean much lower peak concentrations of phosphorylated forms in the erythrocytes throughout the experiment than were found in the muscle at the end. It is tempting to speculate that thiamine transporters such as those in mammalian muscle cells (Dutta et al., 1999); Zhao et al., 2002) also exist in Baltic salmon. The high activity of thiaminophosphokinase, a thiamine phosphorylating enzyme in the skeletal muscle cells of Baltic salmon, might also explain the high TPP concentrations.

The amounts of TTH in the muscle and ovary were calculated on the basis of the relative mass of the tissues and TTH concentrations in them (Expt 2, IV). There appeared to be high amounts of TTH in both the muscle and the ovary. The high amounts in the muscle tissue would comprise a large pool of thiamine for the fish, which is contrary to the principle of daily need and absence of tissue stores of thiamine in mammals (Dollery, 1991).

6.4. Use of thiamine hydrochloride in the prevention of M74 syndrome

Thiamine hydrochloride is the only thiamine compound that has been used in experiments on treating fry showing EMS- or M74-like symptoms or in preventing the appearance of these symptoms in healthy fry. Thiamine hydrochloride is relatively cheap and highly soluble in water. On the other hand, it is acidic in water and the pH of the bath has to be elevated in order not to harm the fry. According to Jansen (1972), thiamine hydrochloride is easily destroyed in neutral water.
These facts have led to the “beside the tank” or “on the river bank” mixing of the thiamine hydrochloride treatment solutions.

Many studies on the use of thiamine hydrochloride lack other criteria of diagnosis of M74 than typical symptoms. The obvious rareness of illnesses other than M74 in late yolk-sac fry of Baltic salmon (III) make it probable that the reported results of a cure for or prevention of M74 symptoms really have treated M74.

6.4.1. Bathing of the yolk-sac fry

The first report of the dose-dependent prevention of M74 syndrome was in the preliminary report of III in Koski et al. (1996). Amcoff et al. (1998a) showed a similar phenomenon even with the treatment of eggs during the water hardening. The dose-dependent prevention of M74 syndrome together with the elevation of the thiamine concentrations above the critical concentrations of M74 development (II, III, Amcoff et al., 1998a) are key facts supporting the role of thiamine deficiency in M74 syndrome. The histopathological lesions in the brain of the sick yolk-sac fry observed by Lundström et al. (1999a) also support this view.

The treatment of the yolk-sac fry for the prevention of M74 requires usually at least two treatments, sometimes more (III, pers. comm. of J. Rytilahti and J. Tulokas). This is more laborious than the injection treatment of the female broodfish. It allows, however, simultaneous monitoring of the natural concentrations of thiamine in an untreated subsample of eggs and fry and the prevention of M74 syndrome in the bulk of production at a salmon hatchery. Another approach to increase the concentration of thiamine in the bathed yolk-sac fry might be to lengthen the treatment time. The absorption of an acidic water-soluble vitamin would also benefit from a lower pH of the bathing water of the fry. This would, however, cause problems with the ionic balance of the fry.

The lack of reports of side-effects in the thiamine hydrochloride treated yolk-sac fry of salmon is in accordance with the results of thiamine treatment in other animals, where extremely high parenteral and systemic doses are required for toxicity (Unna, 1972)

6.4.2. Injection of the female broodfish

The injections of thiamine hydrochloride alone or in combination with astaxanthine prevented M74 syndrome in the offspring of the injected feral females (III). The effect of the i.p. injected thiamine hydrochloride on the TTH of the eggs of the female broodfish was shown to be dose-dependent in Expt 3 of IV. The levels achieved in the eggs at spawning time seem to be relatively independent of the length of the time span between the injection and ovulation. This might be an indication that the incorporation of thiamine into the ovary occurs relatively soon after the injection. According to Smith (1957), the metabolism of ripe eggs in the gonad is slow, which might indicate that the achieved concentrations in the eggs do not decrease to a large extent. The results of IV also show that preventive i.p. doses of ca. 20 mg kg\(^{-1}\) would be enough to prevent the occurrence of M74 in the fry of the feral Baltic salmon. This dose would lead to higher thiamine concentrations in the eggs than the critical levels for M74 development (Löflund et al., 1999; I). The reported doses so far, 100-125 mg kg\(^{-1}\), have been ca. 5-6 times as high as the preventive dose 20 mg kg\(^{-1}\) (Börjeson et al., 1999; III).

The effect of the thiamine hydrochloride injections on the “GSI” of the feral Baltic salmon (IV) is interesting. It might indicate that the thiamine status of the feral female has an effect on the fecundity or the size of the egg of the wild and feral Baltic salmon. The effect of dietary environmental factors on the reproduction success in salmon, and fish in general, is rather limited (see e.g. Wootton, 1991), but Blom and Dabrowski (1995) showed that differences in dietary vitamin C intake led to significant differences in the number and total mass of eggs produced by
rainbow trout. To be sure that thiamine has a corresponding effect in Baltic salmon would require further studies.

The present results do not indicate any harmful effects of thiamine hydrochloride injections on the female broodfish. The pH of the solutions used in III and IV was neutralised, but Börjeson et al. (1999) found no signs of haemorrhage or peritonitis in their study of i.p. injections with acidic thiamine hydrochloride only 2-6 weeks before ovulation. The treatment of female broodfish can be regarded as safe for the fish.

Injections of wild or feral Baltic salmon were not only performed on females that had been caught for fish farms. The i.p. injection of caught and released wild or feral females with thiamine hydrochloride is a practical measure to be used in a small river. In a small reproductive population the effects of M74 syndrome on parr production might be considerable (Jokikokko et al., 1995). While occurring at a high prevalence, M74 syndrome might also cause a bottleneck for the genetic variation in a small salmon river like the Simo. However, the effects of thiamine injections on these factors require further investigation.
CONCLUSIONS

1. M74 syndrome was shown to be common in the feral and wild Baltic salmon of the Bothnian Bay rivers of Finland during the study years 1995-1997. Experiments with yolk-sac fry originating from wild broodfish of the Atlantic salmon River Teno and feral Baltic salmon of River Daugava did not show M74 syndrome in these stocks. The concentrations of TTH in the eggs were higher than the threshold value for the development of M74 syndrome in the Baltic salmon of the Bothnian Bay rivers of Finland. The concentrations of TTH in the white muscle and liver tissue were also higher in the River Teno and Daugava material than in the Bothnian Bay rivers.

Cross-fertilization experiments between female and male salmon of farmed and wild background were performed. For the first time an effect, although small, of the male background on the degree of M74 syndrome was observed. Yolk-sac fry from the crossing between wild males and and wild females suffered greater mortalities than those from farmed males and wild females.

Inter-stock variations in the prevalence of M74 syndrome and thiamine tissue concentrations may be due to the differences in migratory patterns or the length of starvation before spawning in separate Baltic salmon stocks. These probably lead to differences in the supply of thiamine for the female broodfish and thus for the eggs and yolk-sac fry. There may also be regional and temporal differences in the levels of thiaminase activity in the intestinal tract of Baltic salmon females. This could lead to variability between stocks in the oral bioavailability of thiamine. An association has also been found between the annual prevalence of M74 syndrome and concentrations of the 2,3,7,8-tetrachlorodibenzo-p-dioxin toxic equivalent of certain dioxin-like organochlorines. All these factors, and especially the interactions between them, require further research in the future to obtain a comprehensive view of the causes of M74 syndrome.

2. Intraperitoneally-injected thiamine hydrochloride was found to have a relative bioavailability nearly as good as that intra-aortically injected. The absorption from the peritoneal cavity was rapid and resulted in higher concentrations of TTH in the blood of feral Baltic salmon females than in farmed fish. TTH, however, distributed into the eggs and white muscle of the farmed females to a much greater extent than in the feral broodfish. The high TTH concentrations in the eggs and white muscle indicate some kind of intracellular thiamine “trapping” in these tissues. However, the mechanism needs further investigation.

The white muscle tissue was shown to be a potential storage tissue for thiamine in salmon broodfish, which differs from the daily need and absence of tissue stores of thiamine in mammals.

3. Yolk-sac fry suffering from M74 syndrome were shown to respond in a dose-dependent manner to preventive thiamine hydrochloride bathing. Intraperitoneal thiamine hydrochloride injections of feral Baltic salmon females effectively prevented the development of M74 syndrome in their offspring. A dose-response effect was also shown between the dose and the TTH concentrations in the eggs of the injected females. Lower doses than used at present were shown to elevate the concentrations of TTH in the eggs above the threshold concentrations for the development of M74 syndrome. The preventive treatments of the yolk-sac fry or females were regarded safe for the fish.

Intraperitoneal astaxanthine injections may have an effect on the capacity of the fetus or yolk-sac fry to utilize thiamine during development. In view of the beneficial effect of thiamine hydrochloride injection alone, it is not necessary to include astaxanthine in the injection given to the female broodfish in the prevention of M74 syndrome.
The feral female Baltic salmon broodfish that had been injected with thiamine hydrochloride produced a greater mass of eggs than the untreated controls. Determination of the possible effect of the thiamine status of the female on its fecundity or the size of the eggs would require more research.
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