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Philosophiae Doctor (PhD) Thesis 2010:23

Tetradecylthioacetic acid (TTA) – A functional feed ingredient for Atlantic salmon (*Salmo salar* L.): Growth, sexual maturation and health

Tetradesyltioeddiksyre (TTA) – en funksjonell fôringrediens til atlantisk laks (*Salmo salar* L.): vekst, kjønnsmodning og helse

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Sandnessjøen, Desember 2009

Henriette Alne

ABBREVIATIONS

1+	Smolt transferred to sea in spring, more than one year post hatching
0+	Smolt transferred to sea in autumn, less than one year post hatching
ACO	Acyl-CoA oxidase
CMS	Cardiomyopathy syndrome
CPT 1	Carnitine palmitoyl transferase I
GH	Growth hormone
HSMI	Heart and skeletal muscle inflammation
IGF-I	Insulin-like growth factor-I
IPN	Infectious pancreatic necrosis
MCT	Medium chain triacylglycerols
PD	Pancreas disease
PPAR	Peroxisome proliferator activated receptor
TTA	Tetradecylthioacetic acid

SUMMARY

Farmed Atlantic salmon (*Salmo salar* L.) is transferred to sea at different times during the year. Independently of time after sea transfer, the salmon experience a period of low performance, characterized by reduced appetite, feed efficiency, growth rate, condition factor, muscle fat and fat retention during first spring in sea (Paper I). In addition, such low-performing periods may be a predisposing factor for outbreak of diseases, such as infectious pancreatic necrosis (IPN) (Paper III) and heart and skeletal muscle inflammation (HSMI) (Paper IV). The temporary reduction in muscle fat content observed during first spring in sea may indicate a higher demand for available energy than what is obtained from traditional salmon feed. In the present thesis tetradecylthioacetic acid (TTA) is used in both 1+ and 0+ farmed Atlantic salmon, during first spring in sea, to enhance muscle fatty acid oxidation capacity. In contrast to previous studies with TTA in fish feed, the idea with the present work was to use diets supplemented with a low level of TTA and only during short periods, where more energy might be needed.

Dietary supplementation of TTA significantly reduced the frequency of sexual mature 1+ male Atlantic salmon first autumn in sea (Paper II). Compared to control, dietary supplementation of TTA resulted in a three-fold reduction in incidence of sexual mature males (0.6% versus 1.8%). The final body weight was not affected. As muscle fat was reduced by dietary TTA in spring, the effect on maturation is probably a consequence of lower energy status at this time, too low to initiate the maturation process.

In 1+ smolt transferred to sea in spring, a natural outbreak of IPN occurred eight weeks after sea transfer. Relative percent survival for the fish fed TTA in sea water was 70% compared with the un-supplemented groups, significantly reducing mortality from 7.8% to 2.3% (Paper III). Plasma chloride was reduced by dietary TTA and related to increased IPN survival, which

may suggest an improved osmoregulatory capacity in fish fed a TTA-supplemented diet. Reduced fat and enhanced β-oxidation rate were further observed in white muscle following administration of TTA, indicating that TTA resulted in a re-allocation of dietary fatty acids from storage to energy producing oxidation. In addition to the effect on survival during the IPN-outbreak, TTA was found to significantly reduce mortality during a natural outbreak of HSMI in 0+ smolt. The mortality was reduced from 4.7% in the un-supplemented to 2.5% in the TTA-supplemented groups (Paper IV). Expression of several genes related to lipid metabolism (*Peroxisome proliferator activated receptor* (*PPAR*) α and β, *carnitine palmitoyl transferase I* (*CPT I*) and *acyl-CoA oxidase* (*ACO*)) were higher in ventricles from salmon fed TTA. At the same time, urea content in plasma was found to be lower in fish fed TTA. Taken together, these results indicate that the dietary effect on survival may partly be due to an altered metabolic balance, with better protein conservation due to increased lipid oxidation. In addition, 0+ salmon previously fed a TTA-supplemented diet had a higher growth rate during the disease period, compared to the control.

In conclusion, TTA reveals a range of biological and physiological effects in salmon, resulting in better resistance to diseases such as IPN and HSMI, reduced sexual maturation and similar or better growth performance. The presented thesis may exemplify the importance of developing functional feed ingredients in modern fish farming for achieving better growth, reduced sexual maturation and improved health.

SAMMENDRAG

Oppdrettet atlantisk laks (*Salmo salar* L.) blir satt i sjøen til ulike tider av året. Uavhengig av tidspunkt for utsett i sjø gjennomgår laksen en periode kjennetegnet ved redusert appetitt, fôrutnyttelse, vekstrate, kondisjonsfaktor, fettinnhold i muskel og retensjon av fett første vår i sjø (artikkel I). I tillegg kan slike perioder med lav ytelse også være en utløsende faktor for utbrudd av sykdommer, som infeksiøs pankreas nekrose (IPN) (artikkel III) og hjerte- og skjelettmuskel betennelse (HSMB) (artikkel IV). Den midlertidige nedgangen i fettinnhold i muskel om våren kan være forårsaket av et høyere behov for tilgjengelig energi, enn hva som oppnås ved ett tradisjonelt laksefôr. I denne avhandlingen er tetradecylthioacetic acid (TTA) brukt i fôr til både 1+ og 0+ oppdrettslaks første vår i sjø, for å øke kapasiteten for fettsyreoksidasjon i muskel. I motsetning til andre forsøk med TTA i fiskefôr var idèen i dette arbeidet å bruke fôr tilsatt en lavt nivå av TTA og kun i korte perioder, hvor laksen muligens trenger mer energi.

Tilskudd av TTA i fôret gav en signifikant reduksjon i frekvensen av kjønnsmodne hanner av 1+ atlantisk laks første høst i sjø. Sammenlignet med kontrollfôret hadde TTA-fôret laks bare en tredjedel så mange kjønnsmodne hanner (0,6% versus 1,8%) (artikkel II). Sluttvekta i forsøket var ikke påvirket. TTA reduserte mengde fett i muskel om våren. Effekten på kjønnmodning kan sannsynligvis være en konsekvens av redusert energistatus på denne tiden og at denne ble for lav til å starte modningsprosessen.

I et forsøk med vårutsatt smolt (1+) brøt det ut naturlig IPN åtte uker etter sjøutsett. Relativ prosent overlevelse for fisk fôret TTA i sjøvann var 70% sammenlignet med gruppene uten tilsetning, med en signifikant redusert dødelighet fra 7,8% til 2,3% (artikkel III). Nivået av klorid i plasma ble redusert etter fôring med TTA og varierte i takt med IPN dødelighet,

noe som kan indikere at fisk fôret med TTA hadde økt osmoreguleringskapasitet. Etter fôring med TTA ble det også observert mindre fett og et høyere nivå av mitokondriell β-oksidasjon i hvit muskel, noe som indikerer at TTA kan ha resultert i en omfordeling av fettsyrer i fôret fra lagring til energiproduksjon. I likhet med effekten på overlevelse ved IPN-utbrudd ble det funnet signifikant redusert dødelighet ved et naturlig utbrudd av HSMB i 0+ smolt fôret med TTA. Dødeligheten var redusert fra 4,7% i fisk som ble gitt et fôr uten tilsetning, til 2,5% i grupper fôret med TTA-tilsetning (artikkel IV). Sammenlignet med kontrollfisken hadde fisk fôret TTA en oppregulering av flere gener involvert i lipidmetabolismen *(Peroksisom-proliferator aktivert reseptor* (*PPAR*) *α* and *β*, *carnitin palmitoyl transferase I* (*CPT I*) and *acyl-CoA oksidase* (*ACO*)), målt i ventrikkel. Samtidig ble ureainnholdet i plasma funnet å være lavere i fisk fôret TTA. Sett under ett tyder dette på at effekten på overlevelse delvis kan skyldes en endret metabolsk balanse, med bedre proteinkonservering på grunn av økt nedbrytning av lipider. I tillegg viste fisk som tidligere var fôret med TTA høyere vekstrate gjennom sykdomsperioden, sammenlignet med kontrollgruppen.

TTA viser seg å ha en rekke biologiske og fysiologiske effekter i laks som igjen fører til bedre motstandsdyktighet mot sykdommer som IPN og HSMB, redusert kjønnsmodning og like bra eller bedre vekst. Avhandlingen synliggjør viktigheten av å utvikle funksjonelle ingredienser til fiskefôr i moderne fiskeoppdrett for å oppnå bedre vekst, redusert andel tidlig kjønnsmodning og bedre helse.

LIST OF PUBLICATIONS

This thesis is based on the following papers referred to by their roman numerals in the text:

Paper I

Alne, H., Oehme, M., Thomassen, M.S.. Terjesen, B. & Rørvik, K.-A. Reduced growth, condition factor and body energy levels in Atlantic salmon (*Salmo salar* L.), during their first spring in the sea.

Aquaculture Research, re-submitted.

Paper II

Alne, H., Thomassen, M.S., Sigholt, T., Berge, R.K. & Rørvik, K.-A., 2009. Reduced sexual maturation in male post-smolt 1+ Atlantic salmon (*Salmo salar* L.) by dietary tetradecylthioacetic acid (TTA). Aquaculture Research 40, 533-541.

Paper III

Rørvik, K.-A., Alne, H., Gaarder, M., Ruyter, B., Måseide, N.P., Jakobsen, J.V., Berge, R.K., Sigholt, T. & Thomassen, M.S., 2007. Does the capacity for energy utilization affect the survival of post-smolt Atlantic salmon, *Salmo salar* L., during natural outbreaks of infectious pancreatic necrosis? Journal of Fish Diseases 30, 399-409.

Paper IV

Alne, H., Thomassen, M.S., Takle, H., Terjesen, B.F., Grammes, F., Oehme, M., Refstie, S., Sigholt, T., Berge, R.K. & Rørvik, K.-A., 2009. Increased survival by feeding tetradecylthioacetic acid (TTA) during a natural outbreak of heart and skeletal muscle inflammation in S0 Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 32, 953-961.

1. INTRODUCTION

Farmed Atlantic salmon is a very important commodity for Norway, representing an export value of 18.1 billion NOK in 2008 (Norwegian Seafood Export Council, 2009). The salmon farmers are dependent on optimal production, which means good growth, good fish health and low losses during the whole production cycle, to achieve continuous and economical profitable production.

In Norway, the commercial production of Atlantic salmon, *Salmo salar* L., smolt has traditionally been carried out under natural conditions of temperature and photoperiod. This allows sea transfer only during spring. Smolt transferred to sea in the spring, more than one year after hatching, are denoted 1+ smolt. By increasing the rearing temperature and altering the photoperiod (Duncan & Bromage, 1998; Handeland & Stefansson, 2001) it has been possible to produce out-of season smolt transferred to sea during autumn only 8-10 months after hatching. This salmon is denoted 0+ smolt.

Today, different viral fish diseases result in high mortalities and significant losses for the salmon farmers in Norway. If the industry is to remain sustainable, these losses must be reduced. Infectious pancreatic necrosis (IPN) is a widely diagnosed disease, which can cause large losses, both during the juvenile stage and in sea-farmed salmon. Even more focus is, however, now directed to diseases such as heart and skeletal muscle inflammation (HSMI), pancrease disease (PD) and cardiomyopathy syndrome (CMS), affecting larger fish. Among these diseases, only for PD a vaccine is so far available in the market.

It has previously been documented that un-vaccinated Atlantic salmon survival during natural outbreaks of the bacterial disease furunculosis, caused by *Aeromonas salmonicida,*

and cold-water vibriosis, caused by *Vibrio salmonicida*, were strongly affected by dietary treatments (Rørvik *et al*., 2003). Positive effects of high dietary levels of long-chain polyunsaturated fatty acids of the n-3 family (EPA/DHA) were enhanced when combined with low levels of iron. As preventive actions against diseases are a vital part of the modern aquaculture industry, it is appropriate to pose the question whether feed optimisation may have significant effects also in relation to viral diseases. Myocarditis is present in the pathology of HSMI (Kongtorp *et al*., 2004a), PD (Ferguson *et al*., 1986) and CMS (Ferguson *et al*., 1990). During the last years it has been discussed whether these diseases may be a kind of life-style diseases following the increase in dietary fat and in growth rate during the last decades. HSMI was first diagnosed in a farm on the coast of mid-Norway in 1999, but has undergone a serious escalation over the last ten years (Olsen *et al*., 2007). In 2008, the number of farms reporting HSMI was 144 (Johansen *et al*., 2009), compared to 94, 83 and 54 in 2006, 2005 and 2004, respectively (Olsen *et al*., 2007). Both the number of outbreaks and the geographical area affected by HSMI are increasing, but still the highest frequency is in mid-Norway.

Early sexual maturation is another factor leading to losses at some locations, due to negative effects on growth performance, flesh composition, external appearance, behaviour, welfare and survival (reviewed by Taranger *et al*., 2009). Both light manipulation (Oppedal *et al*., 1997; Taranger *et al*., 1998; Porter *et al*., 1999) and restricted feeding (Thorpe *et al*., 1990; Duston & Saunders, 1999) during first winter and spring in sea has been found to reduce the frequency of sexual mature fish during second autumn in sea (grilse). Since the largest or fastest growing fish experience sexual maturation second autumn in sea (Nævdal, 1983; Gjerde, 1984; Duston & Saunders, 1999; reviewed by Taranger *et al*., 2009), some farmers avoid the problem by sorting out the largest fish in

May-June, before secondary sexual characters appear, and hence, no light manipulation is needed (personal observation). Even though maturation in salmon production is most severe after one-sea winter, some farmers also report maturation in male salmon first autumn in sea. This may possibly become a greater problem in the years to come, due to even higher growth rates and high dietary fat content. The methods used for preventing sexual maturation second autumn in sea are not transferable to maturation first autumn in sea. Since the 1+ smolt is transferred to sea on increasing day length during spring, light manipulation is probably not feasible, neither fasting nor restricted feeding. Hence, other methods to reduce sexual maturation have to be investigated.

To assume a more stable and predictable production, a better understanding of seasonal variations may be important. To manage this, future research has to be focused on some special periods during the dynamic production cycle influencing growth performance and the fish's resistance against diseases. Functional feed may be an essential tool in all these aspects, providing the fish with special dietary compounds to improve production and health.

2. GENERAL BACKGROUND

2.1 Tetradecylthioacetic acid (TTA), – a 3-thia fatty acid

The chemical properties of thia fatty acids are very similar to those of ordinary fatty acids, but their metabolism and metabolic effects may differ greatly (Skrede *et al*., 1997). Thia fatty acids have been prepared for a wide variety of purposes, from studying mechanisms of enzyme reactions to preparing non-metabolisable fatty acid analogues or obtaining pharmacological effects (Skrede *et al*., 1997).

Tetradecylthioacetic acid (TTA) is a saturated fatty acid with 16 carbon atoms. The third methylene group from the carboxylic end of the chain is replaced by a sulphur atom as illustrated by the chemical structure in figure 1. When a sulphur atom replaces a methylene group in a carbon chain, only slight alterations in the geometrical structure of the molecule appear (Skrede *et al*., 1997), but the sulphur atom in the β-position of the carbon chain blocks the ability of this fatty acid to be β-oxidized (Berge *et al*., 1989; Hvattum *et al*., 1991; reviewed by Skrede *et al*., 1997; Berge *et al*., 2002).

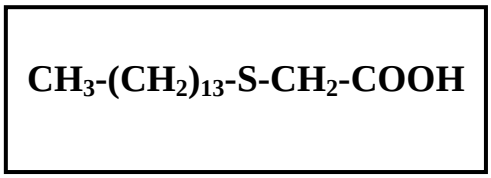

Figure 1. Chemical structure of tetradecylthioacetic acid (TTA).

TTA is a peroxisomal proliferator (reviewed by Bremer, 2001), shown to increase both number and size of peroxisomes and mitochondria in studies with rats (Berge *et al*., 1989). This will in turn result in a higher β-oxidation capacity, as shown in rat livers (Asiedu *et al*., 1993), hearts of rats (Hexeberg *et al*., 1995) and mice (Hafstad *et al*., 2007) and in liver of Atlantic salmon (Moya-Falcòn *et al*., 2004). Results from a study performed by Moya-

Falcòn *et al*. (2006) indicated an increase in peroxisomal β-oxidation capacity, due to the higher activity of acyl-CoA oxidase (ACO) in hepatocytes incubated with TTA at both low (5^oC) and high (12^oC) temperature. In addition, Vegusdal *et al*. (2005) reported liver cells incubated with TTA to secrete less triacylglycerols (TAG) than cells incubated in a control medium, which may indicate a higher ratio of fatty acid oxidation. In rat livers both mitochondrial and peroxisomal β-oxidation was found to be enhanced, and the result was a lower plasma triacylglycerol level (Asiedu *et al*., 1993). Hexeberg *et al*. (1995) reported that treatment with TTA resulted in formation of megamitochondria and increased peroxisomal and mitochondrial β-oxidation with a concomitant reduction of lipid droplets in the cardiomyocytes of rats. They suggested that the increased oxidation rate may be due to the blocked β-oxidation of this sulphur substituted fatty acid analogue. Madsen *et al*. (2002) reported TTA to induce an increase in hepatic fatty acid oxidation and ketogenesis in rats, resulting in a drain of fatty acids from blood and extrahepatic tissues, which again significantly contributes to the beneficial effects of TTA on fat mass accumulation and insulin resistance. However, in studies with cod (*Gadus morhua*) a higher capacity for β-oxidation in liver was indicated, whilst the opposite was suggested for peroxisomal β-oxidation in muscle, since decreased ACO-activity after administration of TTA was observed (Kennedy *et al*., 2007a). In a study with rainbow trout, TTA enhanced the activity of carnitine plamitoyltransferase-I (CPT-I) and ACO in red muscle and liver, whereas no difference to control was found in white muscle, even though the expression of CPT-I was found up-regulated (Kennedy *et al*., 2007b). Hence, TTA seem to have a range of effects in lipid and energy metabolism in different species. To summarize (reviewed by Bremer, 2001); TTA has a hypolipidemic effect explained by series of metabolic effects: Lipoprotein lipase is induced in liver and apoprotein CIII is dowregulated. These effects in the liver will result in a facilitated (re)uptake of chylomicrons and very low density

lipoproteins (VLDL), thus creating a direct transport of fatty acids from gut to liver. As a result, several fatty acid metabolizing enzymes, involved in β-oxidation, ketogenesis and ω-oxidation, are induced and the capacity for fatty acid oxidation is therefore increased.

The effect TTA is shown to have in modulating lipid metabolism seems, at least in part, to be related to its interaction with the members of the peroxisome proliferator-activated receptor (PPARs) family of nuclear receptors. PPARs are lipid-activated transcription factors highly involved in regulatory functions in both development and metabolism (Luquet *et al*., 2004). TTA is a potent ligand for PPARs, observed to activate all subtypes of the receptor (Berge *et al*., 2001; Westergaard *et al*., 2001; Madsen *et al*., 2002). Westergaard *et al*. (2001) reported the human PPARs to be activated in the following order: PPARδ (also known as PPARβ) >> PPARα >> PPARγ, whilst rodent PPAR subtypes were found to be activated by TTA in the ranking order: PPARα > PPARδ > PPARγ (Madsen *et al*., 2002). PPARα activation is observed to give an increase in the peroxisomal enzymes with a modest increase in the mitochondrial fatty acid oxidation in rodent liver and other tissues (reviewed by Khan & Vanden Heuvel, 2003). In addition, Ruyter *et al*. (1997) suggested PPARγ to have an important role in regulating the peroxisomal β-oxidation of fatty acids in Atlantic salmon. Activation of PPARδ has also been reported to promote fatty acid burning in skeletal muscle and adipose tissue in mice (Holst *et al*., 2003; Reviewed by Luquet *et al*., 2004).

In addition to the influence on energy and lipid metabolism, TTA is found to be an antioxidant (Muna *et al*., 1997) and suggested to exert antiinflammatory effects in both mice and humans (Aukrust *et al*., 2003; Fredriksen *et al*., 2004; Dyrøy *et al*., 2005). The antiinflammatory effect of TTA seems to involve both PPAR-dependent and -independent

pathways (Dyrøy *et al*., 2005). In a study with salmon the level of the eicosanoid prostaglandin E_2 (PGE_2) was found to be higher in head kidney macrophages, when TTA was fed at a water temperature of 12^{o}C, whilst an opposite result was obtained when fed at 5^{o}C (Gjøen *et al*., 2007). TTA has also, with success, been experimentally used in dietary treatment of humans in risk of developing life style diseases such as diabetes, and inflammation diseases such as psoriasis (personal communication, Rolf K. Berge).

Negative side effects of PPAR ligands have been observed in relation to human medication (reviewed by Rubenstrunk *et al*., 2007). In farmed salmon, administration of dietary supplemented TTA has led to accumulation of sulphur oxygenated TTA metabolites in the kidneys (Moya-Falcòn *et al*., 2004), changes in kidney morphology (Gjøen et al., 2007), and increased mortality (Moya-Falcòn *et al*., 2004; Kleveland *et al*., 2006).

2.2. Functional feed

Future's fish feed may be defined as a dynamical and functional feed developed to influence the fish physiology and thereby to enhance its ability to adapt to challenges during different stages throughout the life cycle. In some periods the fish may need an extra amount of energy, and during these periods supplementing compounds enhancing its capacity for utilizing the additional energy supplied may be an alternative to the current diets. Until recently, TTA has only to a small extent been tested as supplement in fish feed. In contrast to previous studies with TTA in fish feed, and to minimize negative side effects, our idea was to use diets supplemented with a low level of TTA and only during short periods, where more energy might be needed. The weeks following sea transfer in spring are one such period for salmon, were it changes from freshwater to a marine environment. Several authors have reported fat content to decrease (Jobling *et al*., 2002a,b;

Bendiksen *et al*., 2003; Lysfjord *et al*., 2004) and growth to be suppressed (Usher *et al*., 1991; Duston, 1994; Arnesen *et al*., 1998; Damsgård & Arnesen, 1998; Jobling *et al*., 2002a,b; Bendiksen *et al*., 2003; Toften *et al*., 2003; Lysfjord *et al*., 2004) during the weeks following sea transfer of salmon in spring. The temporary reduction in fat content observed in 1^{+} smolt after sea transfer may suggest that the energy consumption in this period is higher than can be obtained through the energy content in traditional feed. This is further supported by Måsøval *et al*. (1994) reporting spring transferred smolts to reduce their energy stores and condition factor following sea transfer. Beside reducing growth and feed efficiency, such periods may also be a predisposing factor for outbreak of diseases. For instance, IPN outbreaks are most frequently seen about eight weeks after sea transfer (Bowden *et al*., 2002).

As observed for 1+ smolt following sea transfer, the industry experiences a drop in feeding rate for 0+ Atlantic salmon during the first spring in sea (personal communications, Kjell-Arne Rørvik). In addition, outbreaks of HSMI are often seen on 0+ salmon during this first spring period. Whether these periods are caused by the same mechanisms is still not known, but it may seem like at least the 1+ salmon has a high energy demand during this period (Måsøval *et al*., 1994). Different possible ways to achieve more available energy for salmon exist; one way is to increase dietary lipid levels, another is to supply particularly easy oxidizable fatty acids, such as medium chain triacylglycerols, or to enhance muscle fatty acid oxidation capacity by using feed additives like TTA, the latter being the main purpose of this thesis.

3. AIMS OF THE PRESENT THESIS

The present thesis focuses on the low-performing first spring period in the production of both 1+ and 0+ Atlantic salmon and the use of tetradecylthioacetic acid (TTA) as a functional feed ingredient during these periods. In contrast to previous studies with TTA in fish feed, and to minimize negative side effects, our idea was to use diets supplemented with a low level of TTA only during these short periods, where more energy might be needed. The studies were performed at Nofima Marin research site at Averøy, a site frequently experiencing natural outbreaks of IPN (1+) and HSMI (0+) during first spring in sea. The specific aims of the present papers were:

- To investigate feed intake, growth and nutrient retention in 0+ salmon first spring in sea and as for 1+ decide whether any reduction coincides with changes in condition factor and energy status of the fish. A comparison should be made to 1+ salmon after sea transfer, on the same small-scale location and to 0+ in to large-scale sites.

- To evaluate the effects of potentially energy enhancing feed additives (TTA, clofibrate and medium chain triacylglycerols) after sea transfer of 1+ smolt, especially on plasma chloride, muscle fat content and mitochondrial β-oxidation rate in muscle. Further to evaluate whether these supplements might reduce mortality in case of a natural outbreak of IPN in the period after sea transfer in spring.

- To study the response (gene expression, urea content, feed conversion rate and growth) of 0+ smolt to dietary suppelementation of TTA during a possible low-performing period first spring in sea, and further to check whether this supplement might reduce mortality in case of a natural outbreak of HSMI.

4. Overview of the experiments included in the thesis

Four small-scale experiments are included in the thesis, three using 1+ salmon and one using 0+ salmon. In addition two large-scale experiments, both using 0+ salmon, are included in paper I. These two experiments were performed in commercial salmon farms at the coast of mid-Norway and were included to evaluate the results obtained in small-scale experiments. Different data from some of the small-scale experiments are included in more than one paper, as shown in table 1.

Table 1. Year of performing the experiments, scale of the different experiments, whether the smolt used were transferred to sea during spring (1+) or autumn (0+) and in which papers data from the different studies are included.

Year:	Scale :	Smolt:	Papers:
2004	Small	1+	Paper III
2006	Small	1+	Paper I, II and III
2007	Small	1+	Paper I
2007	Small	0+	Paper I and IV
2007	Large (two farms)	0+	Paper I

The small-scale studies were all performed at Nofima Marin research site at Averøy in mid-Norway. At this site a natural outbreak of IPN occurred after sea transfer of 1+ salmon in spring 2004. In addition, a natural outbreak of HSMI was detected during first spring in sea for the 0+ salmon in 2007.

The sexual maturation was studied in the 1+ experiment performed in 2006. In this study the fish were restocked after the different times of TTA administration (Paper II). Only fish representing the average weight within each net pen were restocked and used in the further experimental work. The fish remaining in the original net pens were fed control diet or the TTA-supplemented diet for totally twelve weeks and then all groups were given a high-fat

diet without TTA. Due to restocking of fish with mean body weight only, the 12 week groups consisted of relatively more fish with either high or low body weight, compared to average weight. The 12 week groups were therefore only used in relative comparison.

5. MAIN RESULTS AND DISCUSSION

The presented thesis focuses on different periods in production of 1+ and 0+ Atlantic salmon in sea. The main difference between 1+ and 0+ smolt is the time for sea transfer. 1+ smolt is transferred to sea in spring, more than one year post hatch, whereas 0+ smolt is transferred to sea in autumn less than a year after hatching. The environment is quite different at the time of sea transfer for these two groups. 1+ smolt is transferred to sea at increasing temperature and day length, whereas the 0+ smolt is experiencing both decreasing temperature and day length when transferred to sea during autumn.

Independently of time after sea transfer, both groups seem to experience a low-performing period first spring in sea (Paper I), with reduced growth, depleted muscle fat and reduced condition factor. The decision time for whether the salmon is going to mature the following autumn or not, may be in the spring (Paper II). In addition, the salmon seem to be especially susceptible for diseases, such as IPN in 1+ salmon (Paper III) and HSMI in 0+ salmon (Paper IV), during first spring in sea. The timing for the different topics presented in the thesis is shown in figure 2. In the following sections, main results from the four papers included will be summarized and discussed.

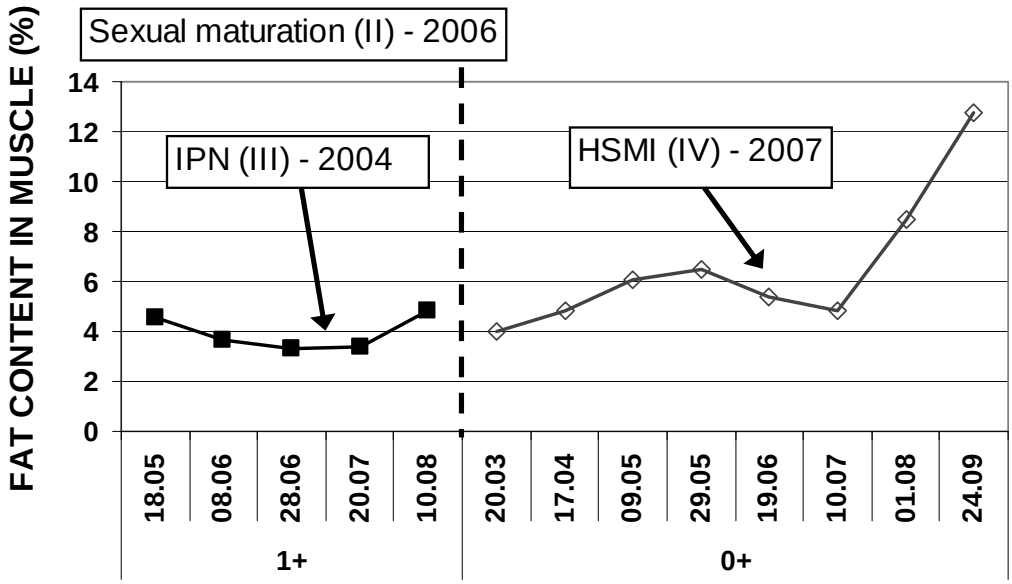

Figure 2. Seasonal variations in muscle fat content first spring in sea for 1+ and 0+ smolt of Atlantic salmon (Paper I). Roman numerals refer to the papers describing the different topics and year of performing the studies. The arrows show the time of the year for the natural outbreaks of IPN and HSMI on 1+ and 0+ salmon, respectively.

5.1 Periods of low performance

Atlantic salmon had, before the present work was initiated, been shown to experience a period following sea transfer in spring of 1+ smolt with reduced appetite and growth (Usher *et al*., 1991; Duston, 1994; Arnesen *et al*., 1998; Damsgård & Arnesen, 1998; Jobling *et al*., 2002a,b; Bendiksen *et al*., 2003; Toften *et al*., 2003; Lysfjord *et al*., 2004), reduced fat reserves (Jobling *et al*., 2002a,b; Bendiksen *et al*., 2003; Lysfjord *et al*., 2004), condition factor and energy reserves (Måsøval *et al*., 1994). Our results (Paper I, II and III) confirms and extends this findings, showing reduced muscle fat content, condition factor, growth rate and poor feed conversion ratio until approximately eight weeks after sea transfer.

No low-performing period has been observed after sea transfer of 0+ smolt in autumn (Lysfjord *et al*., 2004). This is also in accordance to a significant increase in both body weight and length the first four weeks following sea transfer of 0+ Atlantic salmon in November (Stefansson *et al*., 2009). These data were further supported by our results, indicating no drop in fat content and condition factor following sea transfer in autumn (Paper I). The 0+ smolt were, however, found to experience a period during spring similar to what has been described to the 1+ smolt following sea transfer. Also for the 0+ smolt this spring period was characterized by reduced appetite and growth rate, poor feed conversion ratio, depleted fat content, reduced condition factor and low retention of fat (Paper I and IV). The results we obtained in the small-scale study were further validated by data from two large-scale experiments in commercial farms (Paper I), underlining the negative implications for optimal production. Normally, a positive relationship between feed intake and water temperature is observed (Austreng *et al*., 1987), but it seems that in some periods during the production cycle no such relationship exists. The observed drop is obviously not caused by reduced sea water temperature, since it happened in spring with both temperature and photoperiod increasing. Even though this happened at the same time of the year for both smolt types, different factors may have contributed. So far the underlying mechanisms are, however, not fully understood. For the 1+ smolt this period seems to be especially energy demanding (Usher *et al*., 1991; Duston, 1994; Arnesen *et al*., 1998; Damsgård & Arnesen, 1998; Jobling *et al*., 2002a,b; Bendiksen *et al*., 2003; Toften *et al*., 2003; Lysfjord *et al*., 2004), with adaptation from living in freshwater to living in a totally different environment in marine water. In paper II and III we showed that 1+ smolt fed TTA had increased muscle β-oxidation rate and lower muscle fat content nine weeks after sea transfer. At the same time normal plasma chloride level was observed, indicating that the increased capacity to use muscle fat reserves to produce energy may be associated

with improved osmoregulation. Interestingly, this TTA-treated smolt showed significantly higher survival during a natural outbreak of IPN eight weeks after sea transfer, which indicate that a reduced disease resistance might follow osmotic stress. Whether the drop in performance and muscle fat during spring for 0+ salmon is related to osmoregulatory problems still remains unclear, even though no high chloride levels were observed in the few plasma samples collected from this study (unpublished data). As the observed drop happened during spring and coincides with the natural time for smoltification, is it, however, possible to speculate that some hormones associated with smoltification still remains. Ebbeson et al. (2008) measured differences between winter and spring endocrine profiles, suggesting synergestic hormone interactions promoting smolt development, in particular thyroid hormones, growth hormone (GH) and cortisol interactions with melatonin during the scotophase. Plasma GH has been found to increase during the parr-smolt transformation at the time the fish exhibit maximal hypo-osmoregulatory ability (Schmitz *et al*., 1994), and in accordance to increased day length, with the most marked increase from March to June for fish reared at simulated natural photoperiod (Björnsson *et al*., 1995). Both GH (Boeuf *et al*., 1994; McCormick, 1996; Seddiki *et al*., 1996) and cortisol (McCormick, 1996) is found to increase the salinity tolerance and Na^+, K^+-ATPase acitivity of Atlantic salmon and to act in synergy. McCormick (1996) also found insulin-like growth factor I (IGF-I) to act on salinity tolerance, but it was found less effective than GH. Different hormonal treatments have been reported to either improve (e.g. Boeuf *et al*., 1994; Björnsson *et al*., 1995; McCormick, 1996) or inhibit (e.g. Madsen *et al*., 1997) the parr-smolt transformation and the smolts seawater tolerance. Artic charr (*Salvelinus alpinus*) treated with cortisol and GH before a 24 hour seawater challenge test had a higher hypo-osmoregulatory ability compared to the control group (Ojima *et al*., 2009).

Plasma thyroid hormone is suggested also to be involved in changes in body shape during the parr-smolt transformation of coho salmon (*Oncorhynchus kisutch)* (Winans & Nishioka, 1987). Body shape changes during smoltification are characterized by faster length growth compared to weight gain, giving a reduction in condition factor (Sigholt *et al*., 1995). Hormonal regulation may therefore also be a contributing factor to the observed reduction in condition factor for 0+ salmon during spring. In general, length growth seems to be more stable and less sensitive to energy consumption than weight gain. This is also in accordance to our results, showing a relatively stable length increase both in the present studies and in several newly performed studies (personal communication, Kjell-Arne Rørvik). The reduction in condition factor during the spring period was therefore possibly a consequence of a stagnation in weight gain and a stable length growth. In accordance to this, Stefansson *et al*. (2009) reported length to be less affected by feed restrictions than weight for 0+ salmon after sea transfer in November. Starved fish or fish fed every fourth day (25% ratio) had no weight gain during the first eight weeks following sea transfer, but the length increased significantly in both groups, even though the fish was still shorter compared to the groups fed more often than two of four days (50 % ratio). As smoltifications, growth also seems to be under endocrine regulations, involving several hormonal systems. Nordgarden et al. (2005) reported plasma GH to decline and IGF-I and insulin to rise when the growth rate of the salmon increased. They suggested that the lower GH levels in fish with higher growth may be due to metabolic clearance of GH by GH receptor-binding on target cell, resulting in a growth stimulation, both directly and through increased hepatic IGF-I secretion. Future research of the investigated period should also focus on endocrine regulation and interactions among hormonal systems.

Both apparent fat and energy retention were reduced during the spring period for 0+ salmon. This, together with the observation of no change in protein retention, may indicate that the late spring period is energy demanding also for the 0+ salmon, using most of its fat intake for energy consumption. In addition, our results from both 0+ and 1+ salmon revealed muscle fat content to be a good indicator for whole body energy level, at least during the described periods as shown in figure 3. Because of this relationship, the drop in muscle fat content during the specific spring periods in production of both 1+ and 0+ Atlantic salmon at the described fish weights (Paper I) is a good marker for a reduction in total body energy level.

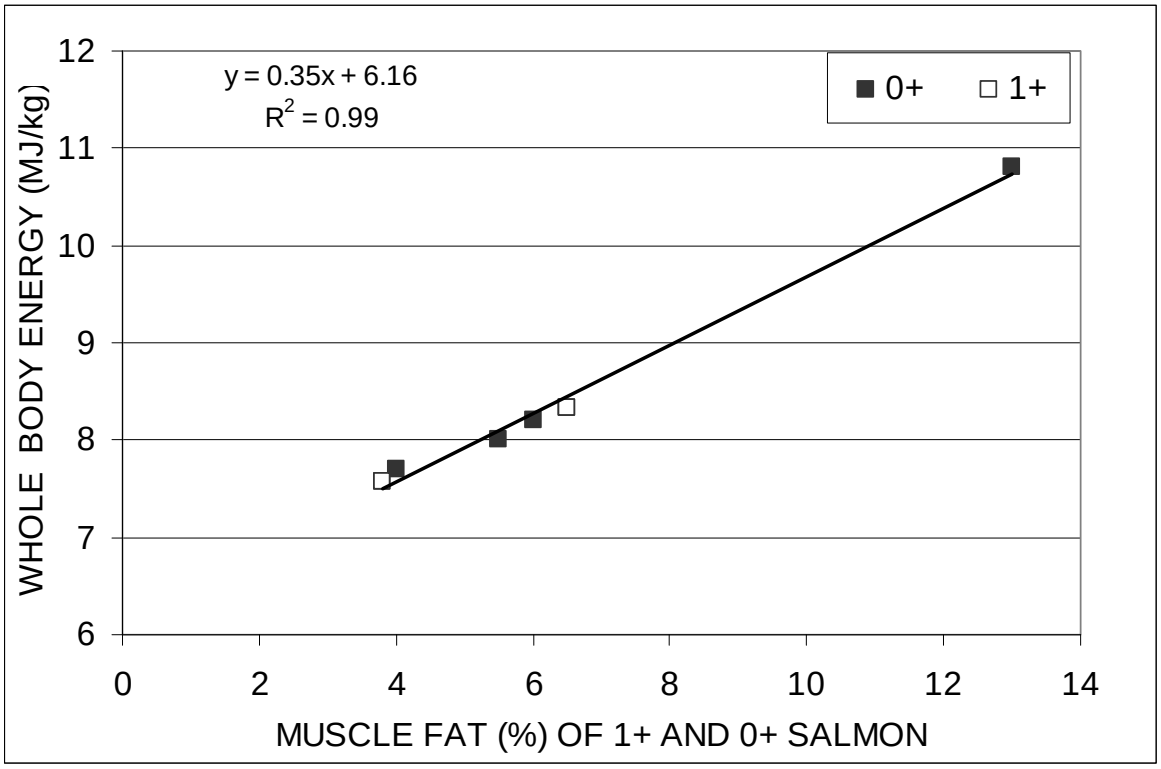

Figure 3. Correlation between whole body energy and muscle fat of 1+ and 0+ salmon during the spring and early autumn period. A highly significant relationship is also observed by leaving out the point of highest level ($y= 0.27x + 6.59$; $R^2=0.98$)

The similarity to the observations on 1+ smolt, including the reduced mortality of the TTA-treated 0+ salmon during the natural outbreak of HSMI (Paper IV), may suggest that also the 0+ smolt may function better by improved lipid oxidation capacity. More research

is, however, needed to clarify mechanisms potentially involved during the spring period for 0+ salmon.

Both smolt groups were found to have a high fat accumulation during late summer and autumn, which is in accordance to other studies both for 1+ salmon (Måsøval *et al*., 1994; Mørkøre & Rørvik, 2001; Roth *et al*., 2005) and for 0+ salmon second autumn in sea (Mørkøre & Rørvik, 2001; Roth *et al*., 2005; Oppedal *et al*., 2006). The retention results (Paper I) revealed great seasonal variations in dietary lipid utilization and fat deposition, and in a much more dynamic way as compared to protein. The five times higher autumn retention for fat in the period of high fat accumulation in the muscle for 0+ salmon, may be due to lipid synthesis also from dietary protein, and/or that protein is used as a substrate for energy metabolism. In 0+ salmon fed TTA during spring, plasma urea content was found significantly lower compared to the control group (Paper IV), indicating a down-regulation of nitrogen excretion. This result may indicate that it is possible to alter the balance between lipid and amino acid metabolism, using feed additives like TTA. Directing lipids for catabolism and energy production may in turn result in higher protein retention and thereby a more economical feed utilization, using the dietary protein for growth.

5.2 Sexual maturation

Several significant factors involved in sexual maturation of Atlantic salmon has been found in a survey performed in New Brunswick and Nova Scotia in Canada (McClure *et al*., 2007). These factors included: fish weight, temperature, size of cages, not feeding any moist feed and feeding intensity. Atlantic salmon often display sexually dimorphic growth, with a pubertal growth spurt in sexually maturing individuals during the spring prior to spawning (reviewed by Taranger *et al*., 2009). This growth spurt in pubertal fish may

initially lead to higher lipid stores than in non-maturing fish, before the muscle is depleted for lipids, proteins and pigments during the later stages of the maturity process (Taranger *et al*., 2009). Both 1+ salmon fed a TTA-supplemented diet and fish fed a control diet experienced a reduction in fat reserves after introduction to sea water, but the reduction was most pronounced in fish fed TTA, becoming significantly lower after nine weeks of feeding. Several authors have suggested that the fish need a certain level of fat or energy stores before the maturation process can start (Shulman, 1974; Kadri *et al*., 1997; Rowe *et al*., 1991). This is in accordance with our results, where TTA both reduced muscle fat stores and reduced the amount of sexual maturing male 1+ salmon. This is further supported by studies performed on male maturation in freshwater where fish maturing is found to have higher body lipid content and mesenteric fat index (MFI) than immature fish (Rowe *et al*., 1991; Shearer & Swanson, 2000). During the study described by Rowe et al. (1991) they provided evidence that maturation in male Atlantic salmon parr is suppressed, when mesenteric fat fails to exceed a certain level by May. Fasting during spring was found to delay increase in total lipids and fat accumulation into the mesenteric store and hence suppress the maturation rate of male parr (Rowe & Thorpe, 1990; Rowe et al., 1991). Rowe & Thorpe (1990) suggested that it may be possible to prevent maturation if the growing season starts late and is too short for the fish to acquire sufficient reserves for spawning. This may also be in accordance with results from other experiments, showing that restricted feeding (Thorpe et al., 1990) or food deprivation during winter and early spring (Duston & Saunders, 1999) reduce the sexual maturation in farming of salmonids. Food intake in the study described by Thorpe et al. (1990) was restricted by feeding every second week over a two-month period, and the incidence of mature fish was lowest when the feeding was restricted from February to March. This is somewhat earlier than the

period when we fed TTA, but the fat accumulation may anyway be delayed long enough to prevent maturation, even though the feed restrictions were performed during late winter.

The sexual maturation observed in our experiment happened the first autumn after sea transfer, while Thorpe et al. (1990) studied maturation one year later. In salmon farming, maturation second autumn in sea happens most frequently and is of greater economical importance, because of the higher fish weight. In a newly performed study, TTA was found to also significantly reduce sexual maturation second autumn in sea for 0+ male Atlantic salmon, one year after sea transfer. The maturation rate was 50% higher in the control group, compared to fish fed a TTA-supplemented diet (personal communication, Regin Arge). Duston & Saunders (1999) also found food deprivation in early winter, late winter, or both early and late winter to significantly reduce the female grilse rate, whereas male grilse rate was reduced by a lesser degree (Duston & Saunders, 1999). In the large scale experiment of the same study food deprivation was found to significantly reduce the grilse rate for both sexes. Food deprivation may result in a lower maturation rate, but it will also influence the growth rate in a period with already low performance. In our study the final body weight on immature fish was, however, not influenced by the different diets, even though in the TTA-groups the fat content in muscle was lower and the maturation rate was reduced first autumn in sea.

A significant relationship between maturation and duration of TTA feeding was observed. Duration of feeding was found to reduce the maturity rate in a curve-linear relationship, with the most distinguished decrease observed as a result of the first weeks of feeding, and then the reduction levelled off. There may be several reasons why prolonged feeding does not seem to give any additional effect on the frequency of maturation. One explanation

may be that the time limit for initiation of the maturation process was exceeded before the last weeks of feeding, and no effect of further feeding will therefore be expected. This may be in accordance with the fat accumulation in both dietary groups between nine and twelve weeks of feeding. Another explanation may be related to a possible threshold level of body fat before the maturation process can initiate. If the fish need to exceed a certain level of fat before maturation can start, the TTA-fed group may already have reduced its fat storage below this level after six weeks of feeding. Hence, no further reduction in relative maturation is expected after prolonged administration of dietary TTA, but as observed, it will then be expected a lowered variance between cages after longer feeding (Figure 4). The reduced fat reserves due to the TTA-supplemented diet may thus, at least in part, explain the lower percentage of maturing fish first autumn in sea.

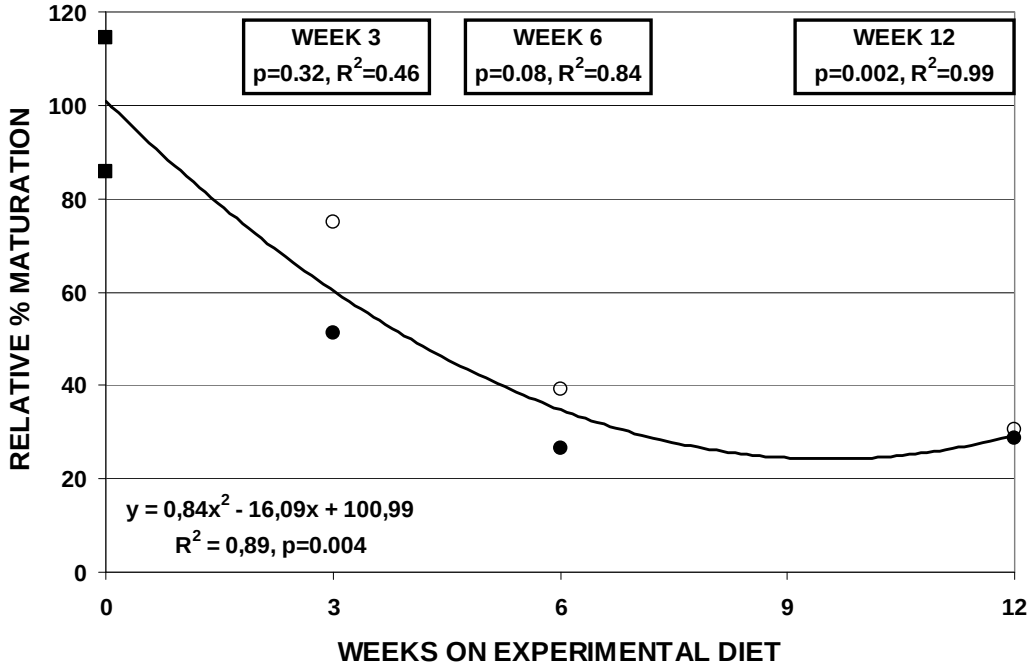

Figure 4. The relationship between weeks on experimental diet and relative percent maturation compared to control. Statistics evaluating the dietary effect within each sampling time (3, 6 and 12 weeks) are shown in the boxes on the top of the figure. Closed and open circles represent the two net pens fed TTA-supplemented diets. Control equal 100%.

In accordance with our results, the decision time for maturation seem to be in spring (Rowe & Thorpe, 1990; Kadri *et al*., 1996; Duston & Saunders, 1999). Since it is suggested that salmon must exceed a certain level of fat or energy reserves in spring before deciding to mature, an interesting speculation may be raised; what will happen with the frequency of sexual maturation (paper II) if the fish do not experience reduction in body fat reserves during spring (paper I). Today, this spring period is primarily considered to be negative for the fish farmers due to reduced growth performance. However, if an elimination of this period becomes possible, will it then consequently lead to a higher frequency of early maturation? If so, losses due to maturation may probably be more expensive for the industry than today's drop in appetite. However, these questions still remain to be investigated, but our results indicate that by using TTA it is possible to both reduce the frequency of sexual maturation (Paper II), as shown for 1+ salmon, and still achieve an equal or even higher growth rate and better feed conversion during the spring period, as observed for 0+ salmon (Paper IV).

5.3 Increased survival during natural outbreaks of IPN and HSMI

The spring period described above also seem to have a high risk for disease outbreaks in production of both 0+ and 1+ salmon. TTA treatment was found to increase survival during natural outbreaks of two severe diseases in Norwegian aquaculture industry: infectious pancreatic necrosis (IPN) and heart and skeletal muscle inflammation (HSMI). Mortality caused by IPN was reduced from 7.8% in the control group to 2.3% in the TTA-supplemented group (Paper III), whereas HSMI mortality was reduced from 4% to 2.5% for fish fed a control diet or a TTA-supplemented diet, respectively (Paper IV).

TTA is reported to act on different biological mechanisms (Reviewed by Bremer, 2001), which may be contributing to the enhanced survival. During the IPN outbreak, fish fed a TTA-supplemented diet was found to have both a reduced fat content and a normal plasma chloride level compared to the control. These results indicate that this fish had managed to use more of its energy stores to improve its seawater tolerance. This was further supported by the approximately three times higher β-oxidation measured in white muscle in TTA-fed smolt. In line with these observations, a higher expression of several genes involved in lipid metabolism was found in fish fed TTA compared to control diet after a natural outbreak of HSMI in 0+ salmon. *Peroxisome proliferator activated receptor* (*PPAR*) *α* and *β* as well as the *carnitine palmitoyl transferase I* (*CPT I*) and the *acyl-CoA oxidase* (*ACO*) were up-regulated in heart tissue of fish fed TTA. This is accordance with results from a study with rainbow trout, showing higher expression of *CPT I* in white muscle in fish fed a TTA-supplemented diet compared to control fish (Kennedy *et al*., 2007b).

As discussed above, our results indicate that osmotic stress may be a predisposing factor for outbreaks of IPN, and perhaps also for HSMI. In line with this, Raynard *et al*. (1991) reported effects of vitamin E level and inclusion of polyunsaturated fatty acids (PUFAs) on frequency of PD and suggested that osmotic stress made the salmon more susceptible to this disease. As for vitamin E, TTA also have some antioxidative properties (Muna *et al*., 1997), and is suggested to exert anti-inflammatory effects in studies with mice and humans (Fredriksen *et al*., 2004; Dyrøy *et al*., 2005). In addition to a lipid regulatory effect, different *PPAR*s are also connected to the regulation of immunological functions in studies with animals and humans (reviewed by Straus & Glass, 2007; Szanto & Røszer, 2008). Several cell types, such as dendritic cells, macrophages, B and T cells and both endothelial and epithelial cells, are reported to be involved, and to be potential targets for the anti-

inflammatory effects of PPAR ligands (Straus & Glass, 2007). Little such information is available in fish, but the level of PGE_2 in head kidney macrophages of Atlantic salmon was found to be influenced by dietary TTA (Gjøen et al., 2007).

During the natural outbreak of IPN there was no reduction in mortality in the group vaccinated against IPN, compared to the group vaccinated with a vaccine without the IPN component (Paper III). Even though only 12% of the Norwegian fish farmers considered the vaccine as an effective tool for reducing IPN mortality (FHL & VESO, 2003), the vaccine companies report increased survival during challenge tests. Higher mortalities are normally obtained during challenge testing than in natural outbreaks post sea transfer. A general stimulation of the non-specific immunological defence mechanisms by certain components in the vaccine, may explain why no effect of vaccination is observed during outbreaks with low to moderate mortalities. In line with this, among post-smolts vaccinated with a bacterial vaccine protective against the diseases *Aeromonas salmonicida* and *Vibrio salmonicida,* Eggset *et al*. (1997) observed significantly higher mortality due to the viral disease IPN in the unvaccinated control fish, compared to the vaccinated group. This indicated that the non-specific immune system was activated by the vaccine and hence, a positive effect of a bacterial vaccine on outbreak of a viral disease, was seen.

The lesions associated with HSMI is partly similar to those described for two other serious diseases in intensive salmon farming; pancreas disease (PD) and cardiomyopathy syndrome (CMS), making it difficult to distinguish between them (Kongtorp *et al*., 2004a,b). Myocarditis is in addition to HSMI also present in diagnosis of PD (Ferguson *et al*., 1986) and CMS (Ferguson *et al*., 1990). Together, these three diseases cause great losses for the global salmon production, leading to high mortalities, reduced growth and in

some cases to reduced product quality. Except for PD, no vaccines are commercially available in the market. Even though a virus inducing HSMI has been isolated (Skjelstad *et al*., 2008), still no vaccines are available for this disease. To the best of our knowledge our study is the first showing any dietary effect on HSMI mortality. Even though TTA has not been tested during outbreaks of PD and CMS, it is possible, and should not be excluded, that this bioactive fatty acid also will improve survival for these viral infections.

Previously, some negative effects of administration of TTA have been reported. In a recent study with mice, cardiac efficiency was found to be markedly reduced in mice treated with TTA, due to a near two-fold increase in the oxygen used for non-contractile processes (Hafstad *et al*., 2007). This seems not to be the case in our study with salmon, since the TTA-supplemented fish actually had a higher survival during HSMI, which is a disease involving significant heart lesions. Dietary supplemented TTA has earlier been found to give changes in kidney morphology (Gjøen *et al*., 2007), as well as an accumulation of sulphur oxygenated TTA metabolites in the kidneys (Moya-Falcòn *et al*., 2004). In our study with TTA-supplemented diets, however, no visual changes in the kidney morphology were observed. Compared to the earlier studies, fish in our study were fed a lower dose for a shorter time, and in a period were the fish is suggested to have a high demand for available energy. In addition, these earlier studies reported lower growth rate and higher mortality using TTA as a dietary supplement for salmon (Moya-Falcòn *et al*., 2004; Kleveland *et al*., 2006), whereas opposite effects were found in the present studies (Paper III and IV). Taken together, it seems that the major negative side-effects observed in earlier work using TTA in feed for salmon may be eliminated by using TTA as a functional supplement only in short periods, were it may help the fish to provide extra available energy by enhancing its ability to oxidize fatty acids. However, further research is

necessary to investigate the potential role of this fatty acid in feed, but at least it shows promising results as a model system for functional feeds to fish. It is, however, important that other possible substitutes are also being investigated. In a recent study, a novel fatty acid (tetradecyl-selenoacetic acid), which in comparison to TTA has a selenium atom instead of a sulphur atom in the third position of the carbon backbone, has been found to have similar effects as TTA in rat livers, being a PPAR ligand with both antioxidative, antiinflammatory and hypolipidemic properties (Dyrøy *et al*., 2007).

5.4 Conclusions and future perspectives

The present thesis shows the potential of using TTA as a functional feed ingredient for fish. A dietary supplementation of TTA was found to give a range of beneficial effects in Atlantic salmon, when used at moderate levels in short specific periods during the seawater phase. In the present studies we used TTA as a dietary supplement during periods with decreasing body lipid level and low growth performance (periods described in Paper I). Our observations revealed TTA to have a significant positive effect on survival during outbreaks of serious diseases such as IPN (Paper III) and HSMI (Paper IV) and also to reduce the amount of sexual mature males first autumn in sea (Paper II). In addition, a higher growth rate was observed during spring for 0+ salmon. Fat storage and energy supply seems to be of importance for the results obtained. Dietary TTA resulted in a lower muscle fat storage, higher β-oxidation of fatty acids and reduced sexual maturation. Further, the fish seemed to be less susceptible to disease outbreaks, perhaps because the fish had more energy available. Taken together, these benefits of using TTA as a supplement in salmon feed may increase the robustness of the salmon.

Our results obtained this far with TTA may work as a model system revealing the importance of functional feed in modern fish farming. A large research programme, partly involved in the work of this thesis, is established to work further with the mechanisms involved in defined periods throughout the production cycle of farmed Atlantic salmon. This way of addressing special periods during life of farmed species by developing specialized (functional) feed, may probably also have a wider relevance in future.

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Paper I

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Reduced growth, condition factor and body energy levels in Atlantic salmon *Salmo salar* L. during their first spring in the sea

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Short running title: Spring growth of post-smolt salmon.

Key words: Salmonids, post-smolt, nutritional status, feed intake

Abstract

We have investigated feed intake and growth in autumn-transferred Atlantic salmon (S0) during their first spring in the sea, a period of low performance in commercial production. We have compared the results with those obtained from spring-transferred smolt (S1), in order to determine whether this reduction in performance is accompanied by changes in nutrient retention, levels of muscle fat, energy status or condition factor. The practical importance of the results obtained in the small scale experiments was evaluated by studies performed at two commercial farms, both using S0 salmon. The feeding rate, rate of growth and degree of feed utilization were low during the first spring in sea, for both S0 and S1 smolt. In both commercial farms the apparent feed intake in S0 was reduced by approximately 50% in the spring. This low-performing period coincided with reduced fat and energy retention, low levels of muscle fat and poor condition factor. Fat retention was reduced from 44.8 (March-May) to 15.4% (May-June) in S0, whereas protein retention did not change, indicating that the energy demand was high during the first spring in sea.

1. Introduction

The commercial production of Atlantic salmon, *Salmo salar* L., smolt has traditionally been carried out in Norway under natural conditions of temperature and photoperiod. This allows sea transfer during the spring, one or more years after hatching. Smolt transferred in the spring following their hatching are denoted "S1" smolt. It has become a trend in recent years to produce increasing numbers of out-of-season smolt by increasing the rearing temperature and altering the photoperiod (Solbakken, Hansen & Stefansson 1994; Thrush, Duncan & Bromage 1994; Berge, Berg, Fyhn, Barnung, Hansen & Stefansson 1995; Duston & Saunders 1995; Sigholt, Staurnes, Jakobsen & Åsgård 1995; Duncan & Bromage, 1998; Duncan, Auchunachie, Robertson, Murray & Bromage 1998; Handeland, Berge, Bjørnsson, Lie & Stefansson 2000; Handeland & Stefansson, 2001). This allows the sea transfer of smolt also during the autumn, only 8-10 months after hatching (such fish are denoted "S0"). The procedure raises the potential for a year-round supply of fresh, marketable fish of consistent size and quality.

Condition factor, level of muscle fat and body energy content all decrease following S1 smolt transfer to the sea in the spring (Måsøval, Åsgård, Wathne, Shearer, Staurnes & Sigholt 1994; Bendiksen, Arnesen & Jobling 2003; Lysfjord, Jobling & Solberg 2004; Alne, Thomassen, Sigholt, Berge & Rørvik 2009a). Furthermore, the S1 smolt may eat little and grow slowly (Usher, Talbot & Eddy 1991; Toften, Arnesen & Jobling 2003; Lysfjord *et al.* 2004), and some loose weight (Bendiksen *et al*. 2003) during the weeks following sea transfer. This period often coincides with outbreaks of infectious pancreatic necrosis (IPN) (Jarp, Gjevre, Olsen & Bruheim 1994; Bowden, Smail & Ellis 2002; Rørvik, Alne, Gaarder, Ruyter, Måseide, Jakobsen, Berge, Sigholt & Thomassen 2007). In contrast to the problems observed for the S1 smolt, S0 smolt maintain a good growth rate after transfer to seawater in the autumn

(Handeland & Stefansson, 2001, 2002). Lysfjord *et al.* (2004) reported that salmon transferred to the sea in September have a higher growth rate than smolt transferred to the sea at two different times in spring. The same study showed also that autumn-transferred smolt deposit body fat during the first month in the sea.

Commercial salmon farmers in Norway have reported that S0 smolt eat poorly and grow slowly during the first spring in the sea (personal communication). The first spring has been studied less deeply than the second spring. Outbreaks of heart and skeletal muscle inflammation (HSMI) often take place during this period (Alne, Thomassen, Takle, Terjesen, Grammes, Oehme, Refstie, Sigholt, Berge & Rørvik 2009b). Periods of slow growth and disease outbreaks are of serious economical importance for commercial fish farmers.

The main objectives of the present study were to investigate growth and feed conversion in S0 salmon during their first spring in the sea and to determine whether any reduction is accompanied by changes in condition factor or energy status of the fish. We have examined feed intake, rate of growth, feed conversion ratio, energy status (measured as whole body energy), the level of muscle fat and retention of fat, energy and protein. We compared the results from S0 smolt with those from S1 smolt transferred to the sea during the spring at the same location. The results from small-scale experiments are supported by results from experiments performed at two different commercial farms, both using S0 salmon.

2. Materials and methods

2.1 Locations and rearing of fish

2.1.1 S0 small-scale experiment

A small-scale S0 experiment was carried out using fish that were hatched in December 2005 (at the Nofima Marin freshwater station at Sunndalsøra, Norway). These were transferred to the Nofima Marin seawater research station at Averøy on the west coast (63 ^{o}N) of Norway in the beginning of November 2006. The mean body weight at sea transfer was 60 grams, and the water temperature was 8.8 ^{o}C. Figure 1 shows the seawater temperature throughout the experimental period. The fish were exposed to additional light (24:0) in the net pens from sea transfer until late June. One underwater light source (400 W) was used in each net pen. The experiment started in late March 2007 and the fish were distributed between two 5 x 5 x 5 m net pens, with 550 fish in each. The fish were fed commercial diets (Biomar, Norway). The mean weight at the start of the experiment was 221 grams, and the standard error of the mean (SEM) was 1 gram. The mean body weight and SEM were 1,585 ± 62 grams at the final sampling in September 2007.

2.1.2. First S1 small-scale experiment (2006)

Fish for the first small-scale experiment with S1 smolt were hatched in December 2004 (at Marine Harvest, Slørdal, Norway). They were transferred in mid-May 2006 to the Nofima Marin seawater research station at Averøy. The seawater temperature at transfer was 9.2^{o}C. The fish were distributed between two net pens (5 x 5 x 5 m), with 1,100 fish in each. The mean body weight at sea transfer was 104 ± 0 grams, and the mean weight in September was of 438 ± 4 grams. The fish were fed a commercial diet produced at the Biomar Technology Centre

(Brande, Denmark). For additional information about the experimental set up and diets, see Rørvik *et al.* 2007; Alne *et al.* 2009a.

2.1.3. Second S1 small-scale experiment (2007)

Atlantic salmon hatched in December 2005 at the Nofima Marin research station at Sunndalsøra, Norway were used in the S1-trial, which was performed in 2007. The fish were transferred to the sea in late May 2007. The seawater temperature at transfer was 8.8 ^{o}C. The fish were distributed on arrival at the Averøy research site between three net pens (5 x 5 x 5 m) with 400 fish in each, and fed commercial diets (Biomar Technology Centre, Brande, Denmark) during the experimental period. The mean weight at the start of the experiment was 107 ± 3 grams, and the mean body weight at the final sampling in September 2007 was 468 ± 42 grams.

The fish in all the small-scale experiments were fed in excess four times per day. Uneaten feed was collected immediately after each meal and pumped up into wire mesh strainers as described by Einen, Mørkøre, Bencze Rørå & Thomassen (1999). Each diet was tested for recovery of dry matter under the environmental conditions present during the experiments as described by Helland, Grisdale-Helland & Nerland (1996), and the weight of uneaten feed recorded was corrected for dry matter losses during feeding and collection.

2.1.4 S0 large-scale studies

The large-scale studies were performed at two different commercial sites in Norway, both situated at 64^{o}N on the coast.

Atlantic salmon S0 were transferred to the sea at the beginning of September at Site 1. The seawater temperature at transfer was 11.5^{o}C. The fish were subjected to artificial light (24:0), using two light sources (240 W and 400 W) in each of two net pens of diameter 120 m from sea transfer until May. Mean number of fish per net pen was 99658 ± 2623. The fish grew from a mean weight of 182 ± 26 grams in October 2006 to 3,382 ± 218 grams in October 2007.

S0 smolt were transferred to the sea in October 2006 at Site 2. The seawater temperature at transfer ranged from 12.5 ^{o}C to 13.1 ^{o}C. The fish were subjected to artificial light (24:0), using an underwater light (1,000 W, 2-3 m depth) in each of two net pens of diameter 157 m from sea transfer until mid-May. Mean number of fish per net pen was 201938 ± 19138. The fish grew from a mean weight of 126 ± 2 grams in November 2006 to 2,907 ± 346 grams in October 2007.

Figure 1 shows the temperature profiles for the two sites during the experimental period. The fish at both sites were fed to satiation using standard commercial feed (Skretting, Norway) in accordance with the manufacturer's guidelines based on fish size. The fish were fed numerous small meals, continually throughout the day. Figure 2 presents the mean specific feeding ratios (SFR) for the two commercial sites, based on data from the production control programme at each farm.

2.2 Sampling and analyses

2.2.1 Small-scale samplings

All fish within a net pen were anaesthetized (MS 222 metacaine, ALPHARMA, Animal Health Ltd., Hampshire, UK, 0.1 g L^{-1}) and bulk-weighed at each time-point for the determination of rate of growth in the small-scale experiments.

Three groups, each of 10 fish,were sampled and killed at the start of the experiment (S0) or at sea transfer (S1). Thereafter, ten fish from each net pen were sampled every third week until August 2007 for S0 and until August 2006 for the first S1 experiment. Fish were sampled every 8 weeks until late September 2007 in the second S1 experiment. Fish were randomly sampled for analyses, representing the average weight. The weight (to the nearest gram) and the length (to the nearest 0.5 cm) were measured for each fish.

The fat content in the Norwegian quality cut (NQC) of the muscle (NS 9401 1994) was determined by diethyl ether extraction in a Soxtec analyzer (Soxtec System HT6, Tecator, Höganäs, Sweden), following HCl hydrolysis (Soxtec System 1047 Hydrolyzing Unit) for the S0 and the second S1 experiment. Samples from the first S1 experiment were analysed using the method described by Folch, Lees & Sloane Stanley (1957). The fat content of one pooled sample per net pen was determined.

The whole body fat content, energy content and protein content were measured at the start of the second S1 experiment in 2007. At all later samplings in this experiment, the fish were separated into carcass, viscera and liver and each component was analyzed separately, before the values for the whole fish were calculated taking body compartment weight into account. Fat

was analysed gravimetrically after extraction with ethyl acetate using the Soxtec system HT6, after extraction in petroleum ether at 100 ºC (viscera S1 July sampling) (Soxtec System HT6), or after acidic hydrolysis and extraction in petroleum ether (Soxtec System 1047 Hydrolysing Unit and Soxtec System HT6) (liver S1 July sampling). Energy content was calculated using a Parr 3000 bomb calorimeter (Parr, Moline, IL, USA). The whole body nitrogen (N) content was analysed using the Kjeltec Auto System (Tecator, Höganäs, Sweden), and the protein calculated as N x 6.25.

2.2.2 Large-scale samplings

Ten fish from each net pen, representing the average weight, were, as described for the small-scale experiment, sampled monthly from October 2006 until October 2007 (Site 1) and from November 2006 to October 2007 (Site 2). The fat content in the NQC of the muscle (NS 9401 1994) was determined by ethyl acetate extraction, in accordance with NS 9402 (1994) for the Site 1 samples and by the Soxtec method (as described above for the small-scale experiment) for Site 2.

2.3 Calculations

The condition factor (CF) was calculated as: $CF = (\text{weight} \times (\text{length}^3)^{-1}) \times 100$. The rate of growth of the fish is presented as the thermal growth coefficient (TGC) (Cho 1992). The TGC incorporates both fish size and temperature and was calculated as: $TGC = (W_b^{1/3} - W_a^{1/3}) \times (\sum T)^{-1} \times 1000$, where W_b is the final weight, W_a is the initial weight and $\sum T$ is the sum of day degrees during the period. The factor of 1,000 is included in order to make the figures easier to handle. The feed conversion ratio (FCR) was calculated as: FCR = (weight of feed eaten) x (final biomass - initial biomass + weight of dead fish)$^{-1}$. The specific feeding ratio (SFR) was

calculated as: SFR= (weight of feed supplied x $((B_0+B_1)/2)^{-1}$) x 100, where B_0 is the initial biomass and B_1 is the final biomass in each interval, as estimated by the production control programme. Figure 2 shows the mean values of SFR for five-week intervals.

Table 1 gives details of the diets used in the 2007 studies of retention. Nutrient retention was calculated as:

Nutrient retention (%) = 100 x (final nutrient content – initial nutrient content) x (nutrient ingested)$^{-1}$ where the nutrient contents and nutrient ingested was calculated as:

Nutrient content = biomass x nutrient content of whole fish (%)

Nutrient ingested = feed intake x nutrient content of feed (%).

The degree of fat retention determined may be higher than the true fat retention because whole body fat can include fats synthesized from non-fat dietary components (*de novo* synthesis).

2.4 Statistical analyses

The data were analysed by simple regression analysis or as one-way ANOVA with fat content in muscle, condition factor, FCR or TGC as dependent variable, and date or week as class variable using the "general linear model" (glm) procedure, using the SAS software package (SAS Institute Inc., 1990). Net pen mean was used as the statistical unit and results are presented as mean ± SEM (standard error of the mean). The level of significance was chosen at $p \leq 0.05$. The proportion of the total variation that is explained by the model is expressed by R^2, and calculated as the marginal contribution of the parameter to the mean square value (Type III sum of squares).

3. Results

3.1 Small-scale experiments

3.1.1 S0 experiment in 2007

The fish initially accumulated fat in the muscle from the start of the experiment in late March 2007, but the fat content then dropped significantly from 6.5 ± 0.2% in late May to 4.9 ± 0.1% in mid-July (Fig. 3a). The fat content of muscle then increased from the sampling in July throughout the autumn. The condition factor followed the same pattern, with a similar significant drop during the spring (Fig. 3b).

The TGC fell significantly during the spring, from 3.86 ± 0.23 in the period late March to mid-May (period of feeding 0-6 weeks) to 1.16 ± 0.06 in the period mid-May to mid-June (period of feeding 6-12 weeks) (Fig. 4), coinciding with the drop in both muscle fat and condition factor. The FCR, in contrast, increased during the same period (Fig. 4). Both TGC and FCR improved during the following two periods (periods of feeding 12-18 weeks and 18-25 weeks, mid-June to September). The change in TGC was significantly correlated with changes in both muscle fat content and condition factor, and the correlations are quantified in Equations (1) and (2), respectively.

(1) $TGC = 1.90 + 0.42 * \Delta F$ $(p=0.026; R^2=0.59)$

(2) $TGC=2.43 + 7.23 * \Delta CF$ $(p<0.011; R^2=0.69)$

where ΔF is the change in muscle fat content (%) and ΔCF is the change in condition factor between subsequent samplings.

3.1.2 S1 experiments in 2006/2007

Both the fat content in muscle and the condition factor were significantly influenced by season in 2006. The level of muscle fat dropped from 4.5 ± 0.0% at sea transfer in mid-May to 3.3 ± 0.2% six weeks later (Fig. 5a). The condition factor followed the same pattern, decreasing until six weeks after sea transfer (Fig. 5b). Both the muscle fat and the condition factor increased significantly from late summer onwards, nine to twelve weeks after sea transfer.

In 2007, as in 2006, both the muscle fat content and the condition factor were influenced by season. There was no statistically significant reduction after sea transfer, but numerically the condition factor fell from 1.19 ± 0.03 at sea transfer to 1.12 ± 0.01 eight weeks later (p=0.13). Both the muscle fat and the condition factor increased significantly from eight to sixteen weeks after sea transfer (condition factor from 1.12 ± 0.01 to 1.24 ± 0.03 and muscle fat content from 3.8 ± 0.15% to 6.5 ± 0.42%).

The rate of growth, measured as TGC, was significantly (p=0.001; R^2=0.94) lower during the first eight weeks after sea transfer (1.28 ± 0.08) than during the next eight weeks (2.32 ± 0.10) for S1 smolt transferred to the sea in the spring of 2007. Problems with the collection of feed waste during the first weeks of feeding meant that it was not possible to calculate accurate FCR values for the first eight weeks in the sea. The value of FCR from 8 to 16 weeks after sea transfer (0.84 ± 0.01) showed that the feed utilization was high.

3.1.3 Body energy and nutrient retention in the small-scale experiments in 2007

Comparing muscle fat content and whole body energy content of the S0 and S1 in 2007 showed a significant and positive relationship between these parameters (body energy = 0.35 x muscle fat percentage + 6.16; $R^2 = 0.9924$).

There was no difference in protein retention between the different sampling time periods for S1 salmon in 2007, but fat and energy retentions were significantly lower during the first period after sea transfer (May-July), than they were during the autumn (Fig. 6a). The retention of dietary protein in S0 was similar for the two first periods (March-May and May-June), but significantly reduced during the period June to September (Fig. 6b). Fat and energy retention differed significantly in all three periods. The retention of dietary fat was approximately three times higher in March-May and almost five times higher in June-September than it was in the period from May to June.

3.2 S0 large-scale experiments

The level of muscle fat fell during the spring in fish at the two commercial farms in mid-Norway (Fig. 7a) in the same way as in the S0 small-scale trial, and this drop was statistically significant at Site 2. Condition factor fell during the spring at both sites (Fig. 7b), and remained low for three to four months (March-June/July). The condition factor increased from July onwards (Fig. 7b), coinciding with a period of rapid increase in fat during the late summer and autumn (Fig. 7a).

4. Discussion

Both the muscle fat and the condition factor dropped significantly during the first spring in the sea in both small-scale and large-scale studies of S0 salmon. Duston & Saunders (1995) reported a significant decrease in the condition factor during the first sea-winter for autumn-transferred smolt, but they were unable to determine the exact time of this fall, since they sampled the fish only in October and May. Likewise, Oppedal, Berg, Olsen, Taranger & Hansen (2006) also reported that the condition factor of S0 smolt fell during the winter and spring. The decrease started as early as January and continued until June. Muscle fat, in contrast, did not fall in the experiments carried out by Oppedal *et al*. (2006). We have sampled the fish frequently in the present study, every third or four week during the experimental period, and this has enabled us to determine relatively precisely the period during which the fat content in muscle and the condition factor decline. The reductions in fat content and condition factor are specific for both S0 and S1 during the first spring in sea, and these reductions in fat content and condition factor do not occur for larger salmon during the second spring in the sea (Mørkøre & Rørvik 2001).

Both the growth factor (TGC) and the feed utilization (FCR) were severely influenced in small-scale experiments on S0, which strongly supports the belief of commercial farmers that S0 Atlantic salmon experience a period of severe reduction in growth and feed utilization during the first spring in the sea, several months after sea transfer. These effects do not coincide with reduced water temperature, and we conclude that other biological factors overrule the normal pattern in which there is a strong positive correlation between feed intake, growth and water temperature (Austreng, Storebakken & Åsgård 1987).

The retention of both fat and energy was also severely reduced during the spring period, in experiments carried out in 2007, for both S0 and S1 fish, with a threefold decrease for fat retention from 44.8 (March-May) to 15.4% (May-June) (S0). This may indicate that the first spring period is energy-demanding for the fish, and they use most of the dietary fat for energy production. This suggestion is supported by the fact that protein retention does not change during the spring. A low retention of fatty acids is associated with a high utilization of these fatty acids for energy production in Atlantic cod *Gadus morhua* (Hansen, Berge, Hillestad, Krogdahl, Galloway, Holm, Holm & Ruyter 2008).

Fat retention varied greatly throughout the seasons, indicating that the utilization and deposition of dietary fat are much more dynamic and depend more heavily on the season than protein utilization, as has been suggested for Atlantic mackerel *Scomber scombrus* (Fjermestad, Hemre, Holm, Totland & Frøyland 2000). Fat retention during the autumn, which is the period of high fat accumulation in muscle, was five times higher than it was in the spring. This may be due to some fat synthesis taking place from dietary protein. Very high values for fatty acid retention indicate that the acids are being synthesized, as they are in Atlantic cod (Hansen *et al.* 2008). In addition, protein might be used as a source of energy, a suggestion that is supported by the fact that protein retention is significantly lower in the autumn than it is in late spring (S0). Several studies on different species have shown that fat and protein retention levels are influenced both by dietary fat levels and the nature of the fat sources (Wang, Liu, Tian, Mai, Du, Wang & Yang 2005; Hansen *et al*. 2008; Torstensen, Espe, Sanden, Stubhaug, Waagbø, Hemre, Fontanillas, Nordgarden, Hevrøy, Olsvik & Berntssen 2008). Moreover, the ratio of dietary protein to energy plays an important role in the utilization of fat and protein (Hillestad & Johnsen 1994). We have used diets with only small variations in fat and protein ratios (Table

1). We conclude that the differences in nutrient retention and deposition between the periods are not a result of the diet composition: they are the result of metabolic changes induced by season-specific signals, such as the light:dark cycle and temperature. Energy metabolism and nutrient utilization vary with the seasons in several species (Fjermestad *et al.* 2000; Hemre & Sandnes 2008) and we suggest that adjusting the diet according to the season can be carried out to maximise feed utilization.

S1 smolt transferred to sea in the spring of 2007 grew slowly during the first eight weeks, and at a faster rate during the subsequent 8 weeks. It has been suggested that osmotic stress may be part of the reason for the slow growth in the weeks after sea transfer (Rørvik *et al.* 2007). Usher *et al.* (1991) showed, however, that the period of suppressed feed intake and growth (up to 30 days) exceeds the period of osmoregulatory adaptation, which is completed within 10 days. Further, Toften *et al.* (2003) found that plasma chloride concentrations are not correlated with feeding rates. This suggests that the low growth rate following sea transfer is not solely a result of osmoregulatory disturbances. We have shown that both condition factor and muscle fat content fall significantly following sea transfer in the spring, as has been shown also in previous reports (Jobling, Larsen, Andreassen, Sigholt & Olsen 2002a; Jobling, Andreassen, Larsen & Olsen 2002b; Bendiksen *et al.* 2003; Lysfjord *et al.* 2004). The retentions of both fat and energy were significantly lower during the first eight weeks after sea transfer than they were in the following eight weeks, and we suggest that this period is energy-demanding for the smolt. Large smolt transferred to the sea in April and medium-sized smolt transferred to the sea in June decrease their body fat percentage during first month in sea, with the most marked decrease taking place in fish that are transferred to the sea in April (Lysfjord *et al.* 2004). In

addition, Måsøval *et al.* (1994) reported that spring-transferred smolt reduce both their energy stores and their condition factor following sea transfer.

The decreases in fat content and condition factor in S1 smolt lasted for one to two months following sea transfer, before these parameters rose again during the late summer and autumn. This timing agrees with results obtained by Jobling *et al.* (2002a, b), in which fat content did not increase until six weeks after sea transfer. Both fat content and condition factor increased rapidly during the autumn, which agrees with earlier results (Måsøval *et al.*, 1994; Mørkøre & Rørvik 2001; Roth, Johansen, Suontama, Kiessling, Leknes, Guldberg & Handeland 2005).

The first sampling in the large-scale experiments took place after only one month following sea transfer. From this time onwards, we observed a gradual increase in fat content for about four months. This agrees with results from Lysfjord *et al.* (2004) showing that the fat content of salmon transferred to the sea in September increases in fillet, viscera and carcass during the first month in sea. In addition, Måsøval *et al*. (1994) showed that the body energy content in smolt increases during the same season, independently of the time after sea transfer. Results obtained by Oppedal *et al.* (2006) also support the conclusion that S0 smolt transferred during the autumn do not experience a low-performance period after sea transfer. Condition factor increases following sea transfer in October, remains stable for a certain period, and then falls during the winter and spring (Oppedal *et al.* 2006). The accumulation of fat during the autumn is rapid also in S1 Atlantic salmon (Måsøval *et al*. 1994; Mørkøre & Rørvik 2001; Roth *et al*. 2005) and during the second autumn in the sea for S0 Atlantic salmon (Mørkøre and Rørvik, 2001; Roth *et al.*, 2005; Oppedal *et al.*, 2006). We have shown that fat and energy retention are significantly higher at the time at which fat accumulates in the muscle, during the late summer

and autumn for both S0 and S1 (Fig. 3a, 5a, 7a). This suggests that both S0 and S1 salmon are evolutionarily programmed to accumulate fat in the muscle during the period in which day length falls in the autumn to prepare for the cold winter season. This is supported by the fact that S1 smolt transferred to the sea in October, half a year after the normal time of sea transfer, show a high growth rate after sea transfer, followed by a period with reduced growth the first spring in the sea (Duncan, Thrush, Elliott & Bromage 2002). Hence, the difference in the patterns of growth and fat deposition between S1 and S0 smolt in the first months after sea transfer can be explained by the difference in the time of sea transfer. The S1 smolt are normally transferred to the sea in the spring when the day length is increasing, whereas the S0 smolt are transferred in the autumn, when day length is decreasing.

To conclude, we have shown in small-scale studies that S0 smolt experience a period of low performance during the spring, 5-7 months after sea transfer. We have confirmed the validity of the small-scale results by studies at two commercial farms, in which the apparent feeding rate fell by 50% during this period. Both S0 and S1 smolt thus experience a low-performance period during their first spring in sea. Levels of fat and energy retention fell, growth slowed, and muscle fat content and condition factor decreased during the weeks after sea transfer for S1 smolt, and after 5-7 months in the sea for S0 smolt. Spring is the natural time for smoltification, and our results indicate that the season and photoperiod are more important causes of the low feed intake and growth than the time of sea transfer.

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Table 1. Proximate analyses of diets used in the study of retention in S0 and S1 Atlantic salmon in 2007.

Macro nutrients	S0		S1	
and Energy	March-May	May - Sept.	May-July	July-Sept.
Dry Matter (%)	93.2	94.5	92.8	92.7
Protein (%)	45.9	42.5	46.7	46.9
Lipid (%)	31.8	29.3	30.2	27.5
Starch (%)	5.1	8.3	9.7	10.6
Ash (%)	9.1	8.2	8.5	8.5
Energy (MJ/kg)	25.0	24.9	25	24.8

Figure legends

Figure 1. Weekly seawater temperature profiles for the two commercial sites (Site 1 and 2) and for the small-scale site during the experimental period for the S0 Atlantic salmon.

Figure 2. Changes in specific feeding rate (SFR) for S0 Atlantic salmon at the two commercial sites (Site 1: solid line and Site 2: broken line) from January to September 2007. For each site SFR is presented as moving average of five following weeks.

Figure 3. Variations in (a) muscle fat content and (b) condition factor for S0 Atlantic salmon during their first spring and summer in the sea in the small-scale experiment in 2007. Significant differences between sampling dates are indicated by different letters on the curves. Variation between net pens within sampling dates is given as standard error of the mean (SEM).

Figure 4. Variations in growth factor (TGC) and feed utilization (FCR) for S0 Atlantic salmon in the small-scale experiment in 2007. Significant differences between sampling periods are indicated by different letters on the curves. Variation between net pens within sampling periods is given as standard error of the mean (SEM).

Figure 5. Changes in (a) muscle fat content and (b) condition factor for S1 Atlantic salmon after sea transfer in the small-scale experiment in 2006. Significant differences between sampling dates are indicated by different letters on the curves. Variation between net pens within sampling dates is given as standard error of the mean (SEM).

Figure 6. Fat, protein and energy retentions measured in Atlantic salmon sampled from the small-scale experiments with (a) S1 and (b) S0 in 2007. The retention is measured in two time periods after sea transfer in the spring for S1 (May-September) and in three time periods first spring/summer in sea for S0 (March-September). Significant differences between sampling periods are indicated by different letters on the bars. Variation between net pens within sampling periods is given as standard error of the mean (SEM).

Figure 7. Changes in (a) muscle fat content and (b) condition factor for S0 Atlantic salmon throughout a year (October 2006 to October 2007) in the sea at two commercial farms in Norway. Upper case letters show significant differences between samplings at Site 1 (solid line), lower case letters show significant differences between samplings at Site 2 (broken line). Variation between net pens within sampling dates is given as standard error of the mean (SEM).

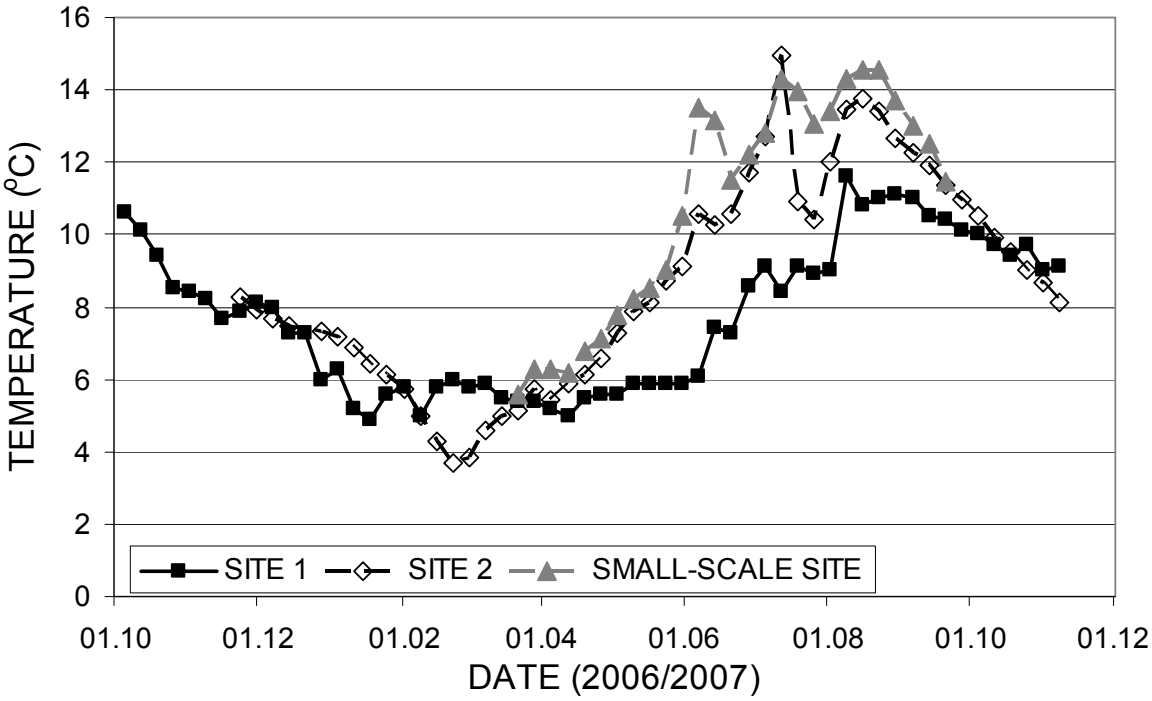
16
14
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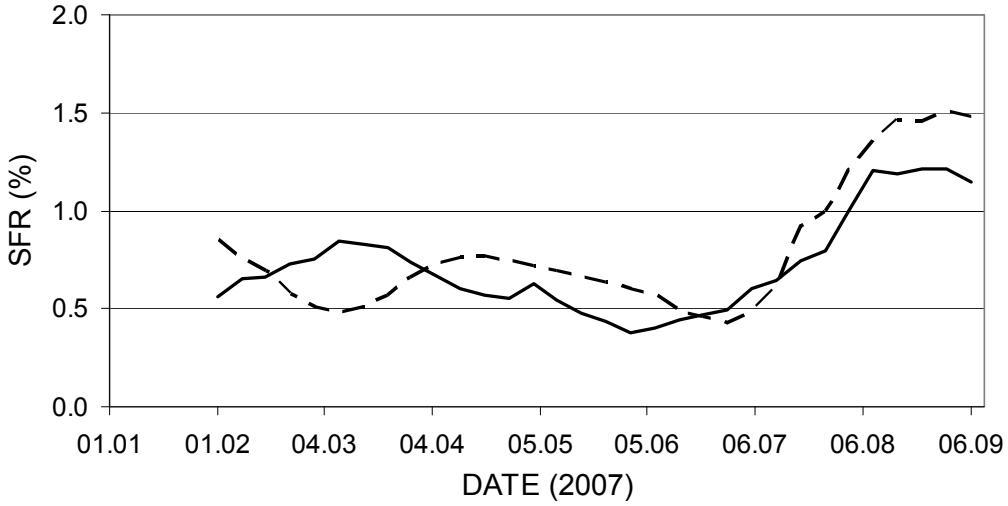
FIGURE 1


2.0
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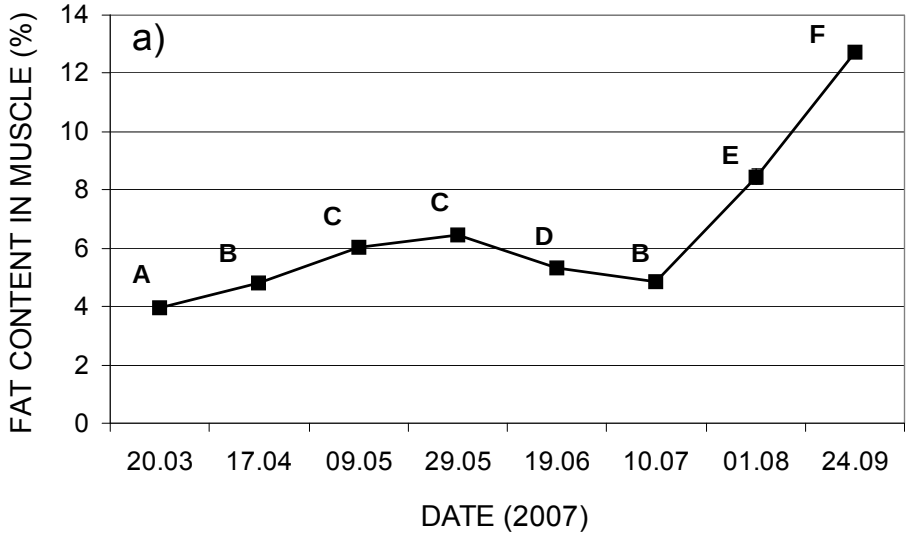
DATE (2007)

FIGURE 2


a)
FAT CONTENT IN MUSCLE (%)
14
12
10
8
6
4
2
0
A
B
C
C
D
B
E
F
20.03
17.04
09.05
29.05
19.06
10.07
01.08
24.09
DATE (2007)

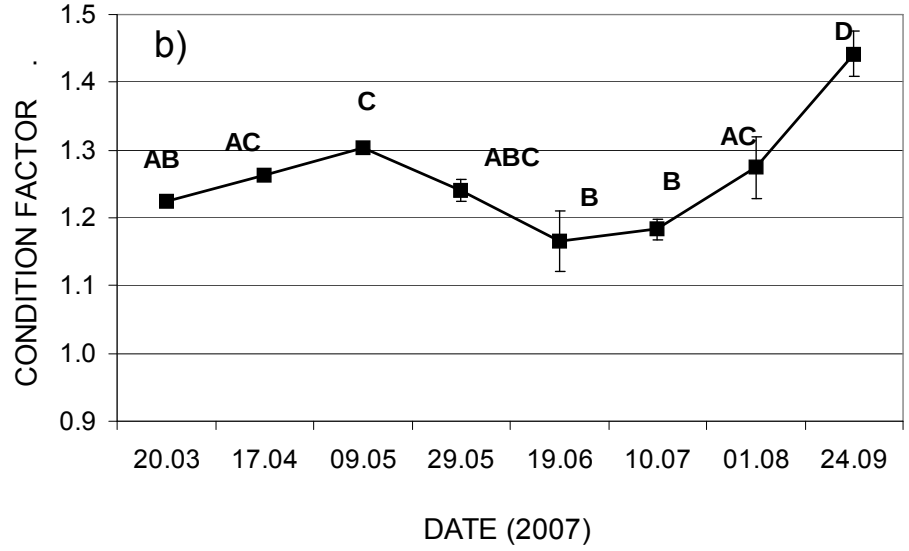

b)
CONDITION FACTOR
1.5
1.4
1.3
1.2
1.1
1.0
0.9
AB
AC
C
ABC
B
B
AC
D
20.03
17.04
09.05
29.05
19.06
10.07
01.08
24.09
DATE (2007)

FIGURE 3

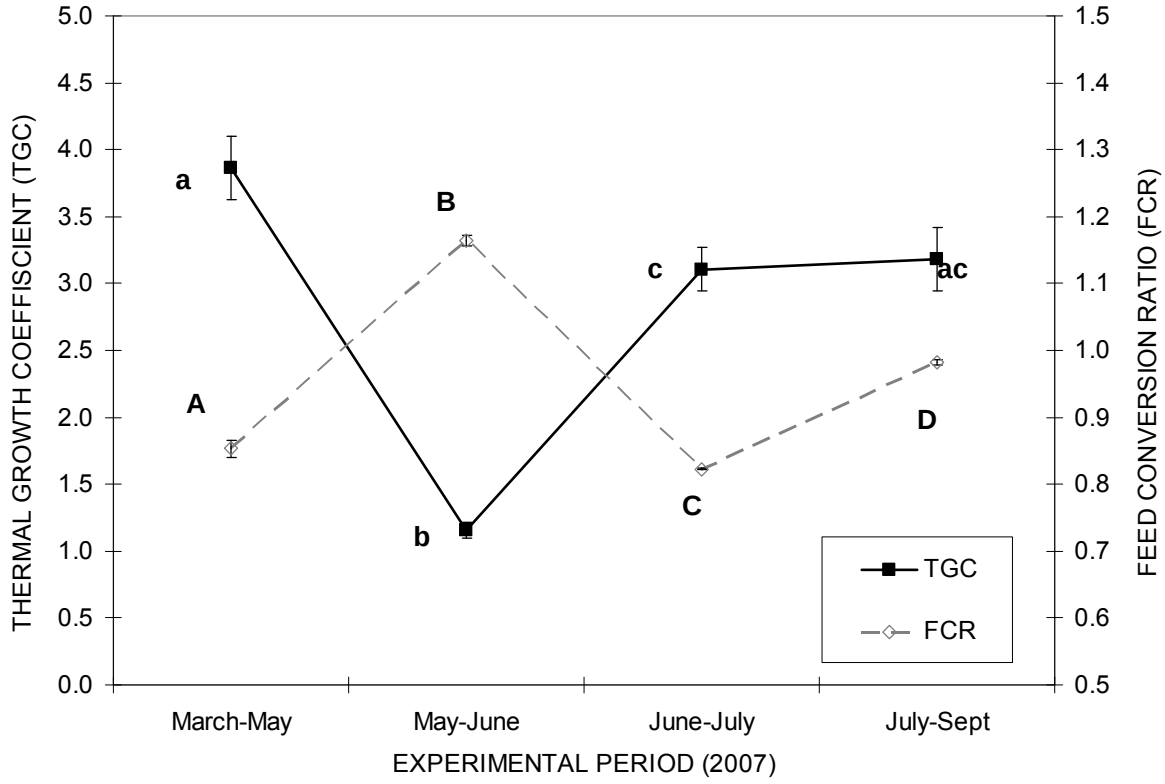

THERMAL GROWTH COEFFISCIENT (TGC)
FEED CONVERSION RATIO (FCR)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
a
b
c
ac
A
B
C
D
TGC
FCR
March-May
May-June
June-July
July-Sept
EXPERIMENTAL PERIOD (2007)

FIGURE 4.

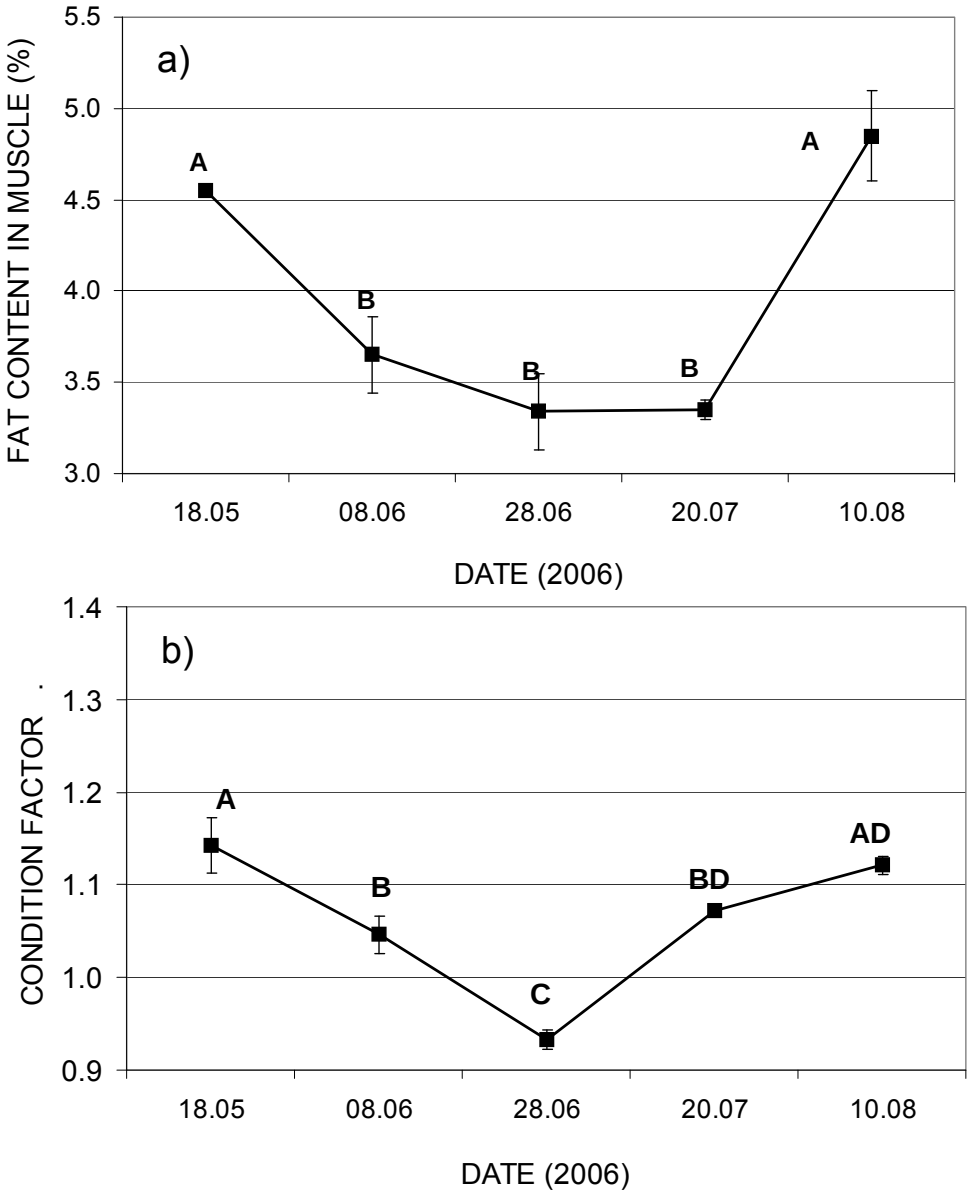

a)
FAT CONTENT IN MUSCLE (%)
5.5
5.0
4.5
4.0
3.5
3.0
A
B
B
B
A
18.05
08.06
28.06
20.07
10.08
DATE (2006)
b)
CONDITION FACTOR
1.4
1.3
1.2
1.1
1.0
0.9
A
B
C
BD
AD
18.05
08.06
28.06
20.07
10.08
DATE (2006)

FIGURE 5

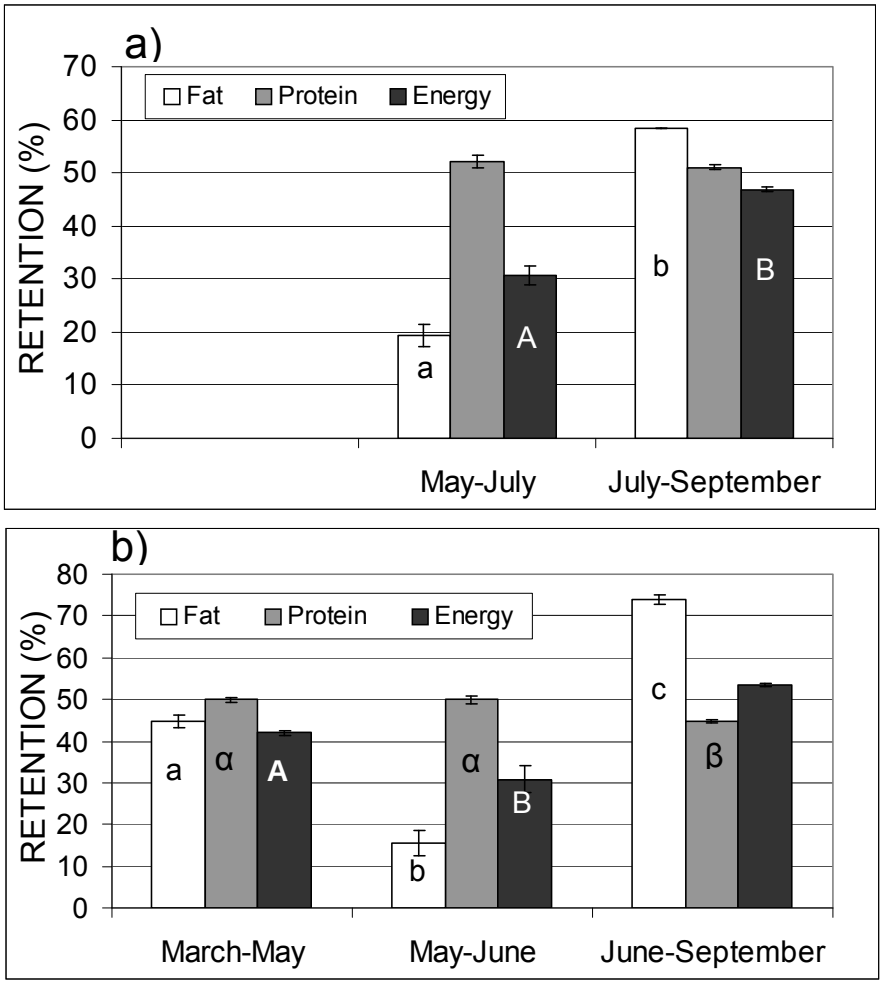

a)
Fat
Protein
Energy
RETENTION (%)
70
60
50
40
30
20
10
0
a
A
b
B
May-July
July-September
b)
Fat
Protein
Energy
RETENTION (%)
80
70
60
50
40
30
20
10
0
a
α
A
b
α
B
c
β
March-May
May-June
June-September

FIGURE 6

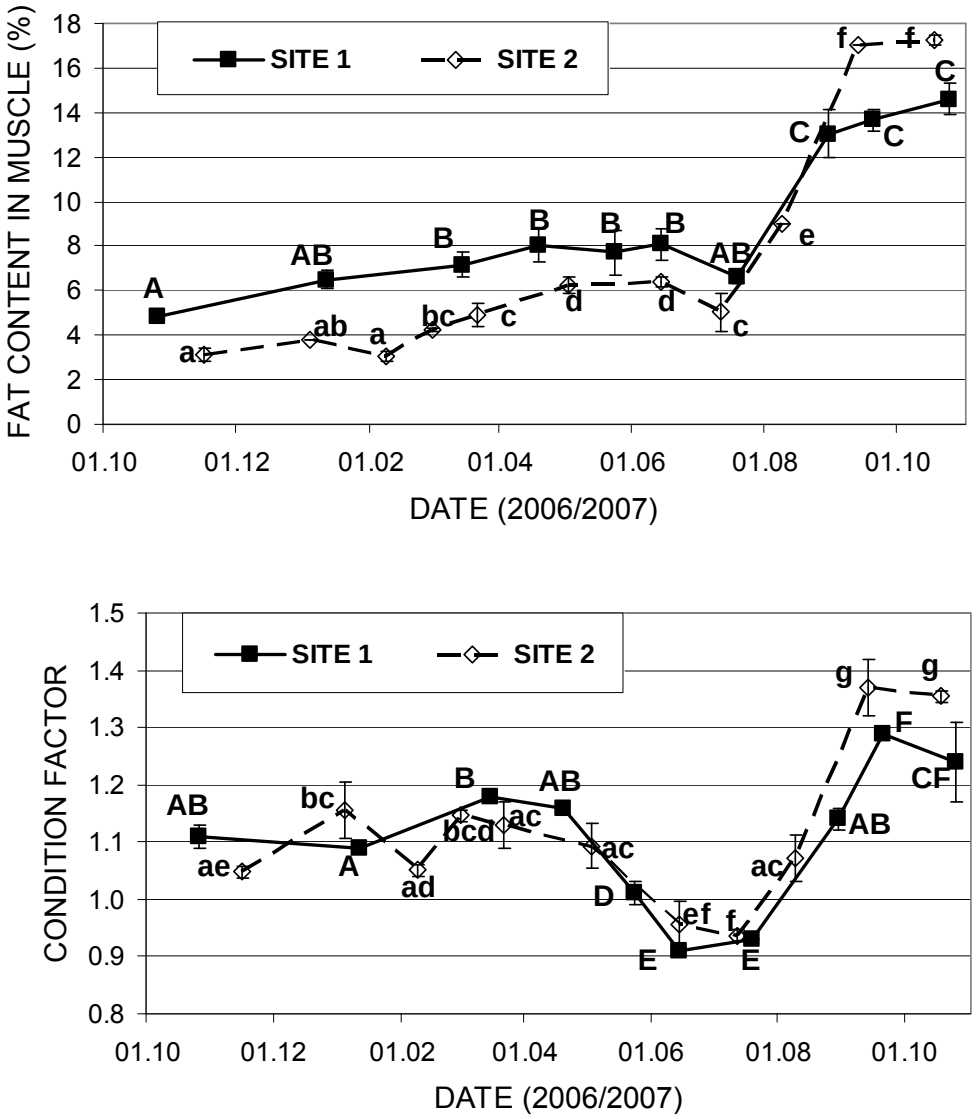

FAT CONTENT IN MUSCLE (%)
SITE 1
SITE 2
DATE (2006/2007)
CONDITION FACTOR
SITE 1
SITE 2
DATE (2006/2007)

FIGURE 7

Paper II

Aquaculture Research

Aquaculture Research, 2009, **40**, 533–541 doi: 10.1111/j.1365-2109.2008.02125.x

Reduced sexual maturation in male post-smolt 1+ Atlantic salmon (*Salmo salar* L.) by dietary tetradecylthioacetic acid

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Abstract

In the present study, the possible effect of dietary treatment on early sexual maturation in post-smolt Atlantic salmon, without any negative effect regarding growth, was investigated. The experiment was performed using 4400 individually marked (Pit tag) 1+ salmon, fed either a control diet or a diet supplemented with 0.5% tetradecylthioacetic acid (TTA) in duplicates for 3, 6 or 12 weeks after sea transfer. Compared with the control, dietary supplementation of TTA resulted in a threefold reduction in incidence of sexual mature males (0.6% vs. 1.8%). A curve-linear relationship between relative reduction in maturation and weeks of feeding TTA was found, indicating that the effect is most marked as a result of the first weeks of feeding and then levelling off. No negative dietary impact on growth was observed. As the level of fat in the muscle was reduced by dietary TTA, it seems that post-smolt supplemented dietary TTA do not accumulate high enough energy stores to start the maturation process, whereas the energy-enhancing effect of TTA due to increased fatty acid oxidation capacity may maintain the growth potential. Compared with immature salmon, sexually maturing fish revealed increased spring growth before the onset of maturation.

Keywords: Atlantic salmon, tetradecylthioacetic acid, sexual maturation, fat

Introduction

Precocious sexual maturation has been, and still is, a problem in intensive farming of several different fish species, such as tilapia *Oreochromis niloticus* (Mair, Abucay, Beardmore & Skibinski 1995; Mair, Abucay, Skibinski, Abella & Beardmore 1997), Atlantic cod *Gadus morhua* (Karlsen, Holm & Kjesbu 1995), Atlantic halibut *Hippoglossus hippoglossus* (Norberg, Weltzien, Karlsen & Holm 2001), European sea bass *Dicentrarchus labrax* (Zanuy, Carrillo, Felip, Rodríguez, Blázquez, Ramos & Piferrer 2001) and the salmonides, for example, rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* (Thorpe, Talbot, Miles & Keay 1990; Kadri, Thorpe & Metcalfe 1997; Porter, Duncan, Mitchell & Bromagea 1999). In commercial salmon farming, sexual maturation results in reduced growth rate (Randall, Thorpe, Gibson & Reddin 1986) and markedly reduced quality during the later stages of the maturity process (Aksnes, Gjerde & Roald 1986). This leads to economical losses for the fish farming industry, and there is a strong focus on developing methods for reducing the maturity rate of farmed fish, preferably without influencing growth rate. Farmed Atlantic salmon may mature sexually in freshwater (parr maturation) (Rowe & Thorpe 1990), first autumn in sea (jack maturation) (Duncan, Thrush, Elliott & Bromage 2002), second autumn in sea (grilse maturation) (Duston & Saunders 1999; Duncan *et al.* 2002) or after two or more sea winters (Duston & Saunders 1999). The term 'early sexual maturation' in commercial on-growing production refers to fish getting sexually mature either during first or second autumn in sea.

If fish are living in stable conditions with abundant food, they should grow rapidly and mature or reproduce as soon as they are developmentally able to do so

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(Policansky 1983; Randall *et al.* 1986). Both biotic and abiotic factors influence this biological process. Intrinsic biological factors influencing the time of first maturity in Atlantic salmon include growth rate, size and age at smolting and the status of the endocrine regulators of development (Randall *et al.* 1986). The external factors influencing these regulators are called extrinsic biological factors and include, for instance, food supply and competition for food.

The levels of fat reserves stored in the fish body are seen to be of importance for maturation. Shulman (1974) reported that the fish must have attained a certain level of fat reserves in the body, whereas Kadri *et al.* (1997) suggested that individuals use a genetically determined threshold fat level as an indicator for cessation of feeding. In addition, several studies have documented an effect of restricted feeding or food deprivation on early sexual maturation both in freshwater (Berglund 1995) and in seawater (Thorpe *et al.* 1990; Duston & Saunders 1999).

In commercial salmon farming, the problem with early sexual maturation is most pronounced in the autumn after one sea winter. Today, photoperiod manipulation is the most common tool for reducing or postponing grilse maturation. Several studies have reported reduced amount of sexually maturing fish after using additional light during first winter and spring (Oppedal, Taranger, Juell, Fosseidengen & Hansen 1997; Taranger, Haux, Stefansson, Björnsson, Walther & Hansen 1998; Porter *et al.* 1999). Porter *et al.* (1999) reported a reduction from 63% maturation in the control group to only 6% maturation in the group exposed to additional lighting. But the use of additional light on salmon in sea tanks is not always found to result in reduced maturity rate (Oppedal, Taranger, Juell & Hansen 1999; Oppedal, Taranger & Hansen 2003), and some studies have even found an increase in maturity rate using continuous or additional light regimes (Kråkenes, Hansen, Stefansson & Taranger 1991; Duncan, Mitchell & Bromage 1999; Endal, Taranger, Stefansson & Hansen 2000; Oppedal, Berg, Olsen, Taranger & Hansen 2006). Hence, the timing as well as the intensity of additional light may be important. The extent of photoperiod manipulation in Norwegian salmon farming is, however, now being reduced (personal observation), due to a generally increased growth rate. Farmers having big fish entering first sea winter may preferably not use additional light. As the largest or fastest growing fish experience grilse maturation second autumn in sea (Nævdal 1983; Gjerde 1984; Duston & Saunders 1999), sorting out the biggest individuals in May or June, before secondary sexual characteristics are noticeable, mainly solve the problem. The advantage of this strategy is to avoid the drop in appetite following onset of light (Endal *et al.* 2000) and utilize increased spring growth before the onset of maturation (Youngson, Wright & Johnstone 1988; Skilbrei 1989).

Whereas a great number of studies have been published on sexual maturation after one sea winter, little information exists on how to reduce jack maturation in Atlantic salmon production. Because of increasing growth rate, jack maturation may possibly become a more significant problem in future. Investigating strategies to reduce this kind of early maturation may therefore be more important in the years to come. As the 1+ smolt is transferred to sea on increasing day length during spring, light manipulation is not feasible, neither fasting nor restricted feeding, as the smolt needs enough feed to maintain the growth potential during the energy-demanding weeks after sea transfer.

Tetradecylthioacetic acid (TTA) is a saturated fatty acid where the third methylene group from the carboxylic end of the chain is replaced by a sulphur atom (Muna, Bolann, Chen, Songstad & Berge 2000). The 3-thia fatty acids cannot be beta-oxidized as the sulphur atom will block the oxidation (Berge, Aarsland, Kryvi, Bremer & Aarsæther 1989; Hvattum, Bergseth, Pedersen, Bremer, Aarsland & Berge 1991; Skrede, Sørensen, Larsen, Steineger, Høvik, Spydevold, Horn & Bremer 1997; Bremer 2001; Moya-Falcón, Hvattum, Dyrøy, Skorve, Stefansson, Thomassen, Jakobsen, Berge & Ruyter 2004). The TTA is a peroxisome proliferator (Bremer 2001), which in studies with rats has been shown to increase both the number and the size of peroxisomes and mitochondria (Berge *et al.* 1989), resulting in increased capacity for beta-oxidation (Asiedu, Skorve, Willumsen, Demoz & Berge 1993). In experiments with Atlantic salmon, TTA was found to increase mitochondrial beta-oxidation in white muscle (Rørvik, Alne, Gaarder, Ruyter, Måseide, Jakobsen, Berge, Sigholt & Thomassen 2007) and liver (Moya-Falcón *et al.* 2004), and resulted in lower body fat reserves (Moya-Falcón *et al.* 2004; Rørvik *et al.* 2007).

This study was performed to evaluate the effect of TTA as a dietary supplement to 1+ smolt in the weeks after sea transfer. Based on previous observations, it was desirable to evaluate whether reduced fat reserves experimentally provoked by TTA (Rørvik *et al.* 2007) might influence sexual maturation in post-smolt Atlantic salmon, without any negative effect on growth.

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Materials and methods

Fish and treatments

The fish used was Atlantic salmon 1 + smolt hatched in December 2004 (Marine Harvest, Slørdal, Norway). The fish was individually marked with Pit tag approximately 3 months before sea transfer. On 19 May 2006, the fish were transferred to Nofima Marin seawater research station at Averøy on the west coast of Norway. Seawater temperature at transfer was 9.2 °C and mean body weight was 104 g. On arrival at the sea site, the fish were randomly distributed in four net pens (5 m × 5 m × 5 m) with 1100 fish in each. The fish were initially fed in duplicate on a control diet or the control diet supplemented with 0.5% TTA (Period 1 – Table 1).

In the main experiment (Fig. 1), 75 fish from each of the four net pens were restocked after 3 and 6 weeks into each of six net pens (5 m × 5 m × 5 m) and fed either a low- or high fat control diet (Period 2 – Table 2) in triplicate until the final sampling in September, 16 weeks after sea transfer. For further information about the main experiment, see Rørvik *et al.* (2007).

In a side experiment, the fish remaining in the four original net pens (initially about 180 fish per pen) were kept on the control or TTA diets until 12 weeks after sea transfer, and then (without restocking) fed the high fat control diet until the final sampling in September. These fish are not directly comparable with those fed for 3 or 6 weeks in period 1, as only fish representing mean body weight were restocked after 3 and 6 weeks. The remaining fish groups had a somewhat different weight distribution (more small and large fish) and as a consequence, the data from the 12-week groups were not included in the overall statistics on absolute growth and sexual maturation. When considering relative per cent maturation, however, the 12-week groups are comparable to those restocked after 3 or 6 weeks.

No signs of diseases were observed during the experiment.

Table 1 Chemical content of diets used during period 1

Experimental diets	Fat (%)	Protein (%)	DM (%)	Ash (%)	NFE (%)	Additives
Control	28.1	45.6	92.6	8.7	10.1	
0.5% TTA	27.2	47.2	94.2	9.0	10.9	0.5% TTA‡

Also described by Rørvik *et al.* (2007).
‡TTA was obtained from Thia Medica, Bergen, Norway.
DM, dry matter; NFE, nitrogen-free extracts, TTA, tetradecylthioacetic acid.

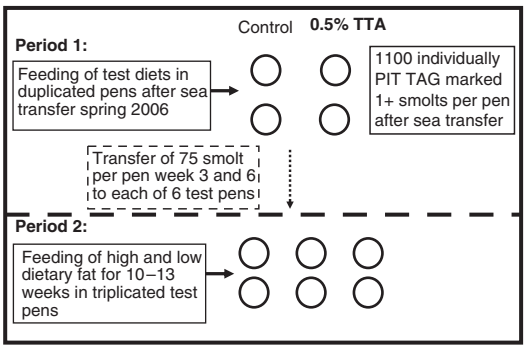

Figure 1 Schematic overview of restocking in the main experiment of fish from each of the pens in period 1 to six pens in period 2. All pens in period 2 contained 2 × 75 fish from each of the four pens in period 1.

Table 2 Chemical content of the low- and high-fat diets used during period 2

Experimental diets	Fat (%)	Protein (%)	DM (%)	Ash (%)	NFE (%)
Low fat	25.1	36.7	92.8	10.9	20.2
High fat	31.6	38.2	93.7	9.2	14.7

DM, dry matter; NFE, nitrogen-free extracts.

Sampling and analyses

Three times 10 fish were randomly sampled at sea transfer, and 10 fish from each net pen, representing the average weight, were randomly sampled after 3, 6, 9 and 12 weeks on experimental diets in period 1. Fat content in muscle [(NQC), NS 9401 1994] was analysed on pooled samples according to Folch, Lees and Sloana Stanley (1957). No overall gender determination of the experimental fish was performed. In the September sampling, all fish were visually graded into mature or immature fish based on external morphological features, such as skin coloration or kype in male salmon. The skin coloration was evaluated on all fish directly after collection from the net pens. The colour was much darker on mature fish and milt was leaking out of them all. Hence, only male fish were found to be mature at the sampling in September. The grading was performed in outdoor light conditions by an experienced technician who did not know which experimental group the fish belonged to during period I. All fish were anaesthetized (MS 222 metacaine, ALPHARMA, Animal Health Ltd, Hampshire, UK. 0.1 g L^{-1}) and fish sampled for fat measurements were killed by a blow to the head.

In the main experiment, live body weight (to the nearest gram) was registered for all fish at restocking

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from period 1 to period 2 and at the final sampling in September, 4 months after sea transfer. The initial individual weight was therefore measured with a 3-week difference for the fish fed for 3 or 6 weeks during period 1. In the side experiment, initial weight was measured after 6 weeks of feeding experimental diets in period 1 and at the final sampling in September.

All diets were analysed gravimetrically for dry matter (DM) after drying at 105 °C (16–18 h), and ash (flame combustion followed by 3–4 h at 550 °C). The content of crude proteins was calculated as N × 6.25 using the semi-micro-Kjeldahl method (Kjeltec-Auto System; Tecator, Höganäs, Sweden), and crude lipid after diethyl ether extraction in a Soxtec analyser (Tecator) after HCl hydrolysis (Stoldt 1952). Proportion of nitrogen-free extract (NFE) was estimated as NFE = DM − (fat+protein+ash).

Statistical analyses

All statistical analyses were performed using the SAS software package (SAS Institute 1990). Data were analysed using one-way analyses of variance (ANOVA) or simple and multiple regression analysis. Experimental diet, weeks of feeding TTA and level of dietary fat in period 2 were used as treatments. Experimental units were net pens. Proportion of the total variation explained by the model is expressed by R^2 and calculated as the marginal contribution of the mean square of the parameter (type III sum of squares). Significant differences among variables within treatments were indicated by least-square means (lsmeans) comparison. As the frequency of observed sexual maturation was low, tests for significant dietary effects were also performed on arcsine transformed data (Zar 1984). The level of significance is chosen at $P \leq 0.05$ and unless otherwise is stated, the results are presented as lsmeans ± SEM (standard error of the mean).

Results

Maturation

In total, 75 fish were classified as sexually mature at the sampling in September. There were 35, 25 and 15 sexual mature fish in the3-, 6- and 12-week groups respectively. Notably, in the 12-week groups, the total number of fish was lower.

Levels of dietary fat in period 2 were not found to significantly influence the proportion of maturing fish during first autumn in sea (1.08 ± 0.26% and 1.16 ± 0.22% for high- and low-fat diet respectively).

As no significant effect of dietary fat during period 2 was found, results from the two dietary fat levels were pooled. When evaluating absolute values of per cent maturation, no significant effect of duration of feeding experimental diets for 3 or 6 weeks in period 1 was observed. However, tests within the diets revealed a trend ($P = 0.14$, $R^2 = 0.74$) to reduced male maturation in fish fed TTA-supplemented diets for 6 weeks (0.60 ± 0.12%) compared with 3 weeks (1.21 ± 0.23%). A similar trend was found using transformed data ($P = 0.13$, $R^2 = 0.76$). No such trends ($P = 0.87$, $R^2 = 0.02$) could be seen in fish fed the control diet for 3 or 6 weeks (1.93 ± 0.50% and 1.82 ± 0.36% respectively). As no significant effect of the time of experimental feeding was observed, data for these groups were pooled, and taken together, the dietary supplementation of TTA for 3 and 6 weeks in period 1 then showed significant reduction in sexual mature males in September ($P = 0.02$, $R^2 = 0.60$) (Fig. 2). The same significance was observed using the transformed dataset.

In the side experiment, feeding the diet supplemented TTA for 12 weeks was also found to significantly reduce the incidence of mature males independently of using the original ($P = 0.002$, $R^2 = 0.99$) or the transformed ($P = 0.001$, $R^2 = 0.99$) dataset (Fig. 2).

When mean relative maturation of fish fed control diet for all durations (3, 6 and 12 weeks) were considered as an estimate of 'zerotime treatment', a significant relationship between maturation and time on experimental diet was observed (Fig. 3). A squared regression line was best fitted to the data showing a

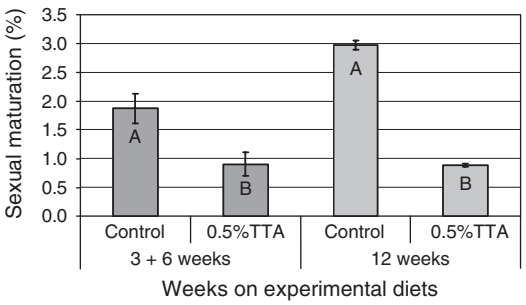

Figure 2 Mean frequency of sexually mature males at the sampling time in September (16 weeks after sea transfer) for salmon fed experimental diets for 3 and 6 weeks, or 12 weeks after sea transfer. The experimental diets are further described in Table 1. Significant differences between dietary groups within samplings (mean of 3 and 6 weeks, or 12 weeks of feeding) are indicated by different letters on the bars and variation is given as standard error of means (SEM).

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rapid decrease in maturation due to the first weeks of TTA-feeding, levelling off after 6–12 weeks. Relative per cent maturation was reduced to approximately 30% of control in the group fed TTA-supplemented diets for 6 weeks. Although the relative per cent maturation was not further reduced by using TTA for 12 weeks, the variation between replicates was smaller and the effect became more significant. A trend to a negative relationship between cage variation and increasing duration of experimental feeding was observed ($P = 0.12$; $R^2 = 0.96$, regression model: SD = 20.7 − 1.6 × weeks of feeding).

Fat content

Mean muscle fat content at sea transfer was 4.6 ± 0.03%. All smolt groups showed reduced level of body lipids during the first weeks after sea transfer

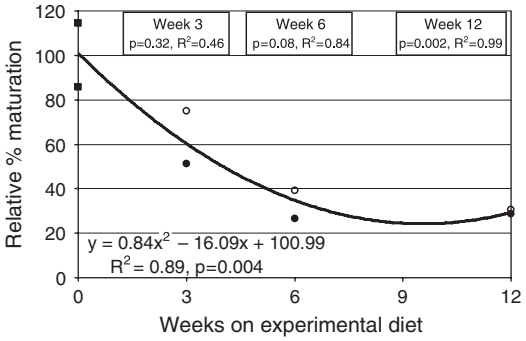

Figure 3 The relationship between weeks on experimental diet and relative per cent maturation compared with control diet. Closed squares (■) represent the estimated zero point (see text), open circles (○) and closed circles (●) represent fish from each of the two net pens fed dietary tetradecylthioacetic acid (TTA) during period 1. A curve-linear regression line was best fitted to the data with the corresponding equation given at the bottom. Statistics evaluating the dietary effect within each sampling time (3, 6 or 12 weeks) are shown in the boxes on the top.

Table 3 Fat content in muscle (NQC) measured after 3, 6, 9 or 12 weeks after sea transfer on a control diet or on a diet supplemented with TTA

Experimental diet	3 weeks	6 weeks	9 weeks	12 weeks
Control	4.2 ± 0.4%	3.6 ± 0.0%	3.4 ± 0.1%	4.9 ± 0.3%
0.5% TTA	4.4 ± 0.2%	3.5 ± 0.1%	3.0 ± 0.1%	4.2 ± 0.5%
P-value	0.75	0.51	0.03	0.31
R^2	0.06	0.24	0.94	0.48

TTA, tetradecylthioacetic acid.

(Table 3). The strongest reduction was observed in groups fed TTA, significantly different from control after 9 weeks of feeding, dietary effects explaining 94% of observed variation in fat content at this sampling. After 12 weeks in sea, fat content was increasing in both dietary groups.

Growth

Mean body weight for all sexually immature fish at the sampling in September was 438 ± 4 g. No statistically significant differences were observed in absolute growth from restocking to the sampling in September among smolts fed the different experimental diets: neither between smolts fed for 3 or 6 weeks in period 1, nor between the groups fed on low- or high fat diets during period 2. Similarly, no significant dietary effect on body weight was observed for fish fed the experimental or control diets for 12 weeks. The growth pattern was, however, found to differ between mature and immature fish (Table 4). Fish becoming mature in the autumn had significantly higher body weight at the sampling in June, but the growth until September was found to be lower.

Discussion

Dietary TTA supplementation to Atlantic salmon smolt during the first weeks after sea transfer significantly reduced male sexual maturation first autumn in sea and without any overall negative impact on growth. In the present study, it was found possible to achieve up to three times reduction in maturity rate of post-smolt Atlantic salmon using TTA as a dietary supplement. The frequency of jack maturation observed in the present study is relatively low compared with the rates observed in studies with parr (e.g. Rowe & Thorpe 1990) or grilse (e.g. Thorpe *et al.* 1990). Although jack maturation may be a greater problem in the years to come, almost 2% losses due to maturation still means undesirable economical consequences for the salmon farming industry.

The higher maturity rate found in the control fed for 12 weeks compared with the control fed for 3 or 6 weeks (Fig. 2) is most likely due to a higher bodyweight variation in these groups, and consequently, a higher percentage of large fish. As only fish representing the average was restocked after 3 and 6 weeks, the largest fish remained in the 12-week group. Larger fish are reported to mature earlier than their smaller counterparts, at least regarding grilse

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Table 4 Body weight (BW), thermal growth coefficient (TGC) and condition factor (CF) at restocking in June and at the sampling in September for the 3- and 6-week groups

	3 weeks of experimental feeding				6 weeks of experimental feeding			
	Mature fish	Immature fish	*P*-value	R^2	Mature fish	Immature fish	*P*-value	R^2
BW in June (g)	141 ± 3.0	108 ± 0.8	<0.001	0.95	176 ± 13.4	114 ± 0.8	0.004	0.78
BW in September (g)	439 ± 23.6	437 ± 0.8	0.91	0.002	404 ± 13.7	439 ± 2.8	0.05	0.51
TGC	1.48 ± 0.09	1.69 ± 0.01	0.06	0.48	1.29 ± 0.13	1.95 ± 0.01	0.002	0.81
CF in June	1.13 ± 0.01	1.07 ± 0.01	0.003	0.80	1.17 ± 0.03	1.00 ± 0.00	<0.001	0.88
CF in September	1.22 ± 0.01	1.25 ± 0.00	0.13	0.33	1.19 ± 0.02	1.24 ± 0.00	0.03	0.55

maturation (Nævdal 1983; Gjerde 1984; Duston & Saunders 1999). This is further supported by our study, where the fish becoming mature had a higher body weight and condition factor at the sampling in June, compared with their immature counterparts.

In the present study, as well as in earlier studies (Moya-Falcón *et al.* 2004; Rørvik *et al.* 2007), TTA is found to reduce body fat reserves in Atlantic salmon, which seems to be important for whether the fish decide to mature or not. The fat reducing effect of TTA in salmon is further supported by a newly performed trial revealing gradually reduced muscle fat by increasing dietary TTA content (not published). Results from a study with Atlantic salmon in freshwater showed that male parr becoming sexual mature had higher total lipid content and mesenteric fat index (MFI) than fish not becoming sexual mature at this stage (Rowe, Thorpe & Shanks 1991). This is also in accordance with a study performed on male chinook salmon *Oncorhynchus tshawytscha* in the freshwater period, where the percentage of maturing males was significantly influenced by body lipid level (Shearer & Swanson 2000). Reduced incidence of sexual maturation in the present study may therefore, at least partly, be explained by the effect TTA is found to have on body fat reserves. Rowe and Thorpe (1990) suggested that fish are adaptive, preventing maturation when the growing season starts late and the duration is too short for fish to acquire sufficient reserves for spawning. The hypothesis that fish need a certain level of energy reserves before the maturation process could begin, is supported by results showing that food restrictions or fasting during spring time reduce sexual maturation in salmon both in freshwater (Rowe & Thorpe 1990; Rowe *et al.* 1991; Berglund 1995) and after one sea winter (Thorpe *et al.* 1990; Duston & Saunders 1999). If the body fat reserves need to exceed a certain relative level before the fish manage or decide to mature, as suggested by several authors (Shulman 1974; Rowe *et al.* 1991; Kadri *et al.* 1997), a dietary supplement, such as TTA, which reduce the fat level in the critical period for this decision, may accordingly reduce sexual maturation.

The duration of TTA-feeding was found to reduce the maturity rate in a curve-linear relationship, with the most marked decrease observed as a result of the first weeks of feeding, and then the reduction levelling off. The present study was, however, not designed to evaluate whether this is due to the duration or the timing of feeding. Fat content in muscle was found to decrease in both dietary groups until 9 weeks after sea transfer (Table 3), but the decrease was most pronounced in TTA-fed smolts. This general decrease in fat content might explain why the proportion of mature fish was also low in the control group. In the present study, as also reported by Rørvik *et al.* (2007), the reduction in muscle fat for the TTA group was found more extensive when TTA was fed for 9 weeks as compared with TTA fed for 6 weeks. When the period for accumulating fat is too short, the fish will not manage to deposit enough energy stores for starting the maturity process, and a longer period of TTA feeding may consequently reduce the maturity rate even more. However, the fat content in salmon-fed experimental diets was increasing in all groups between 9 and 12 weeks of feeding. In salmon, shorter day length has been assumed to provoke marked fat accumulation in the autumn (Mørkøre & Rørvik 2001), and may overrule the fat reducing effect of TTA. This suggests that the salmon has already decided whether to mature or not, before the time of fat accumulation. Feeding TTA during this period, therefore, does not give any additional effect on maturation. This might explain why a further reduction was not observed in relative maturation between fish fed control diet and fish fed TTA-supplemented diet for 12 weeks, compared with fish fed the same diet for 6 weeks, as seen from the curve-linear regression line presented in Fig. 3. There might also be additional reasons why further relative reduction was not

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found. If the fish need to exceed a certain level of fat before deciding to mature, the fat content might have been lower than this level already after 6 weeks of feeding TTA. Hence, no further reduction in relative maturation would be expected after prolonged feeding. In the present study, the cage-dependent variation in relative reduction in maturation, however, seems to be reduced by prolonged feeding. The variation coefficient indicates an approximately 10% reduction per week of feeding.

Dietary level of fat in period 2 did not influence the incidence of maturation in our study. This may further support the hypothesis that the decision for maturation was already taken during the TTA-feeding period in May–June. Enhanced level of dietary fat later on will then not influence this decision. The TTA-feeding for 12 weeks did not give a further reduction in relative maturation compared with 6 weeks of feeding (Fig. 3). Seen in the light of results from earlier studies with salmon maturing during second year in sea, this supports our suggestion for the decision time. The period from April to June is the time when both body lipid levels (Kadri, Mitchell, Metcalfe, Huntingford & Thorpe 1996) and condition factor (Duston & Saunders 1999) are reported to have the fastest increase for fish maturing the coming autumn. This is also in accordance with results from a study performed on Atlantic salmon parr, where the maturity rate was found to decrease when the feeding was restricted in April, May or June (Rowe & Thorpe 1990). The decision time, therefore, seems the same for fish maturing as parr, fish maturing during first autumn in sea (jack maturation) and after one sea winter (grilse maturation).

In the present study, dietary TTA supplementation reduced the maturity rate without influencing growth. This is important, as strategies used for reducing grilse maturation usually results in poorer growth. Use of additional light in sea cages during winter is reported to be followed by reduced feed intake and growth rate for several weeks after onset (Endal *et al.* 2000; Oppedal *et al.* 2006). Because of lower condition factor, initial growth depression after onset of additional light was also suggested by Oppedal *et al.* (1997). Also, restricted feeding is found to reduce growth rate in deprived periods (Duston & Saunders 1999). Earlier studies have reported reduced growth rate in fish fed TTA (Moya-Falcón *et al.* 2004), but our study suggests that using TTA as a dietary supplement for shorter periods and in accordance with the fish needs for energy supply, growth depression is avoided. This is further supported by results from a newly performed trial, indicating even elevated growth rate following TTA supplementation after sea transfer (not published).

Increased spring growth before the onset of maturation in salmon has previously been observed in salmon after one sea winter (Youngson *et al.* 1988). This complies with the documentation of significantly different growth rates in the present study between mature and immature fish first spring in sea. Consequently, the increased weight gain in spring seems to be general for maturing salmon.

To conclude, a dietary supplementation of TTA resulted in up to threefold reduction in incidence of sexually mature post-smolt male Atlantic salmon. The relationship between relative reduction in maturation and weeks of feeding TTA was found to be curve-linear with the most pronounced decrease in maturation following the first weeks of feeding. The observed effect on maturation is probably due to TTA reducing the fish body fat reserves, resulting in too low energy status to start the maturation process. Importantly, and most probably, due to the energy-enhancing effect of TTA by increasing fatty acid beta-oxidation capacity, the good growth potential was maintained using TTA as a dietary supplement. If similar results might be obtained with salmon second year in sea, TTA supplementation might give significant positive economical results. Whether or not TTA-feeding would also suppress grilse maturation still remains to be investigated.

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Paper III

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Does the capacity for energy utilization affect the survival of post-smolt Atlantic salmon, *Salmo salar* L., during natural outbreaks of infectious pancreatic necrosis?

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Abstract

If osmotic stress and reduced seawater tolerance are predisposing factors for infectious pancreatic necrosis (IPN) outbreaks in farmed Atlantic salmon, increased survival by enhancing access to energy would be expected. The aim of the present study was, therefore, to increase energy access in 1-year old Atlantic salmon after sea transfer by increasing the level of dietary fat, by exchanging some of the dietary oil with more easily oxidized medium chain triacylglycerols, or by dietary supplementation of potentially energy enhancing additives such as clofibrate and tetradecylthioacetic acid (TTA). A natural outbreak of IPN occurred 8 weeks after sea transfer, and a significant dietary effect explaining 76% of the variation in mortality was observed. Relative percentage survival for the fish fed TTA in sea water was 70% when compared with the unsupplemented control, reducing mortality from 7.8 to 2.3%. Muscle fat content and plasma chloride were related to IPN mortality, suggesting that reduced hypoosmoregulatory capacity might be a predisposing factor to the onset of an IPN outbreak. Based on the observation of a threefold increase in white muscle mitochondrial fatty acid oxidizing activity by TTA, it is suggested that TTA has resulted in a re-allocation of dietary fatty acids from storage to energy producing oxidation.

Keywords: Atlantic salmon, B-oxidation, energy, IPN, osmoregulation, TTA.

Introduction

Infectious pancreatic necrosis (IPN) is a widespread disease in salmonid fish in many parts of the world (Biering 2002), and has been, and still is, a great problem in the Norwegian fish farming industry (FHL & VESO 2005). In 1995 the financial loss caused by IPN was approximately 350–400 million NOK (Biering 2002). In mid Norway 76% of the farms were affected by the disease in 2000 (Johnsen 2002). A vaccine against IPN was introduced in 1995 (FHL & VESO 2005). Vaccine companies reported good results in challenge tests (Intervet Agenda 2003); however, only 12% of Norwegian fish farmers consider the IPN vaccine effective in reducing outbreaks (FHL & VESO 2003). Consequently, the efficacy of the vaccine in post-smolts is uncertain.

The IPN virus (IPNV) is virulent not only to salmonids such as Atlantic salmon, *Salmo salar* L., rainbow trout, *Oncorhynchus mykiss* (Walbaum), and brown trout, *Salmo trutta* L., but also to a number of other species (Mortensen, Hjeltnes, Rødseth, Krogsrud & Christie 1999; Bruno

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2004). In Atlantic salmon IPN occurs in the freshwater period, but in recent years outbreaks have increased also after sea transfer (Krogsrud, Håstein & Rønningen 1989; Smail, Bruno, Dear, McFarlane & Ross 1992; Jarp, Gjevre, Olsen & Bruheim 1994; Stangeland, Høie & Taksdal 1996; Bowden, Smail & Ellis 2002). Experiments with Atlantic salmon smolts transferred to sea in spring (1^+) vs. smolts transferred in autumn (0^+), have shown higher mortality due to IPN among 1^+ smolts (Bruno 2004). Due to the seemingly low effect of the IPN vaccines, experiments have been conducted to reduce the natural outbreaks of IPN in 1^+ smolts after sea transfer (FHL & VESO 2003). Jarp *et al.* (1994) showed that stress-related transportation affected negatively the frequency of IPN outbreaks after sea transfer. In an experiment by Leonardi, Sandino & Klempau (2003) with dietary additives to improve the immune system, rainbow trout given feed added nucleotides were shown to have a lower IPN mortality than fish fed the control diet.

Seasonal variation in energy stores, growth and feed utilization has been documented in both 1^+ and 0^+ farmed salmon during the grow-out phase (Mørkøre & Rørvik 2001). To manage the transfer from fresh water to sea water, salmon go through a complex and energy demanding smoltification process. This involves changes in morphology, physiology (Eggset, Mortensen, Johnsen & Sommer 1997; O'Bryne-Ring, Dowling, Cotter, Whelan & MacEvilly 2003) and in lipid metabolism (Sheridan 1989; Tocher, Bell, Dick, Henderson, McGhee, Michell & Morris 2000). After sea transfer, smolts have reduced energy stores seen as low levels of glycogen in liver and muscles, and reduced amounts of fat in the abdominal cavity and muscle (Staurnes, Sigholt & Reite 1992). Experiments have shown that smolts have a lower fat content compared with fish remaining in fresh water (Usher, Talbot & Eddy 1991). Salmon smolts transferred to sea water during spring have also been found to reduce their energy stores even further in the weeks after transfer (Måsøval, Åsgård, Wathne, Shearer, Staurnes & Sigholt 1994).

We have previously documented that Atlantic salmon survival during a natural outbreak of furunculosis, caused by *Aeromonas salmonicida*, and cold-water vibrosis, caused by *Vibrio salmonicida*, was strongly affected by dietary treatments (Rørvik, Dehli, Thomassen, Ruyter, Steien & Salte 2003). Positive effects of high dietary levels of long-chain polyunsaturated fatty acids of the n-3 family (EPA/DHA) were enhanced when combined with low levels of iron. Based on cumulative mortalities relative percent survival (RPS) for the high EPA/DHA-low iron group was 70% during an outbreak of furunculosis and 96% during an outbreak of cold-water vibriosis. As preventive actions against diseases are a vital part of the modern aquaculture industry it is appropriate to pose the question whether feed optimization may have significant effects also in relation to viral diseases such as IPN.

The temporary reduction in fat content observed in 1^+ smolts after sea transfer suggests that the energy requirement in this period is higher than can be obtained through the energy content in traditional feed. Besides reducing growth and feed conversion such critical and stressful periods may also be a predisposing factor for outbreaks of disease. Possible ways to achieve more energy are: to increase dietary lipid levels, to supply particularly easily oxidizable fatty acids or to enhance muscle fatty acid oxidation capacity. By feeding fish medium chain fatty acid triacylglycerols (MCT) the regulation point of carnitine-palmitoyl-transferase I and II in mitochondrial membranes can be circumvented. Short and medium chain (10 carbon atoms or shorter) fatty acids can readily penetrate the inner mitochondrial membrane and are thus not dependent on the presence of carnitine or the CPT-I/CPT-II system (Schulz 1991). MCT included in salmon diets have been shown to be highly digestible (Røsjø, Nordrum, Olli, Krogdahl, Ruyter & Holm 2000). In a wide range of animal species different compounds are known to induce peroxisomal and/or mitochondrial β-oxidation enzyme activities. Clofibrate (Clo) has been used to reduce plasma fat in humans (Yang, Kostecki, Calabrese & Baldwin 1990; Bremer 2001) and is found to increase peroxisomal β-oxidation in rodents by up to 20 times (Bremer 2001). Tetradecylthioacetic acid (TTA) is a sulphur-substituted fatty acid that increases mitochondrial and peroxisomal β-oxidation in rats (Madsen, Guerre-Millo, Flindt, Berge, Trondstad, Bergene, Sebokova, Rustan, Jensen, Mandrup, Kristiansen, Klimes, Staels & Berge 2002). Different species react to different degrees: mouse and rat are the most responsive, while primates, guinea-pig and hamster respond weakly or not at all (Reddy & Lalwani 1983; Orton, Adam, Bentley, Holloway & Tucker 1984; Lock, Mitchell & Elcombe 1989). Not much is known about responses in fish, but trout seem to behave in

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a way similar to that of primates. Feeding fibrates or partially hydrogenated fish oils results in a mild increase in the activity of peroxisomal β-oxidation (Henderson & Sargent 1984; Yang *et al.* 1990; Donohue, Baldwin, Leonard, Kostecki & Calabrese 1993; Scarano, Calabrese, Kostecki, Baldwin & Leonard 1994). Furthermore, clofibric acid and bezafibrate, administered to salmon hepatocytes in culture, resulted in a 1.7-fold increase in the activity of acyl-CoA oxidase (Ruyter, Andersen, Dehli, Farrants, Gjøen & Thomassen 1997). Peroxisomal β-oxidation seems to dominate in the liver of juvenile salmon (Frøyland, Lie & Berge 2000), while in muscle mitochondrial β-oxidation is dominating. However, the ratio between mitochondrial and peroxisomal β-oxidation in salmon at difference sizes and life stages is not fully documented. Recently, we have shown that TTA also leads to increased mitochondrial β-oxidation in salmon liver (Moya-Falcón, Hvattum, Dyrøy, Skorve, Stefansson, Thomassen, Jakobsen, Berge & Ruyter 2004).

Based on the previous observations, the main objective of the present study was to evaluate whether such potentially energy enhancing additives (TTA, Clo and MCT) might reduce IPN mortality in 1^+ Atlantic salmon smolts after sea transfer in the spring.

Materials and methods

Experiment 1

Fish and treatments in fresh water

Atlantic salmon 1^+ smolt were kept in duplicate tanks (2 m^3) for 5.5 weeks in fresh water (from 2 April to 11 May 2004) at the AKVAFORSK research station at Sunndalsøra on the west coast of Norway. Mean initial body weight of pre-smolts was 38 g and all fish were individually marked with PIT tags to make salmon in sea water traceable to freshwater treatments. In total 12 tanks and 800 smolts per tank were used with a mean water temperature of 9 °C.

One-third of the smolts were vaccinated with Norvax Compact 4 (*Aeromonas salmonicida* ssp. *salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum* serovars O1 and O2) and two-thirds with Norvax Compact 6 (*Aeromonas salmonicida* ssp. *salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum* serovars O1 and O2, *Vibrio viscosus* and a surface protein from IPNV). Intervet Norbio AS delivered both vaccines.

All diets (also in sea water) were manufactured by high-pressure moist extrusion by EWOS Innovation AS (Table 1). During the freshwater period fish in eight tanks were given control feed (F1), in two tanks control feed was supplemented with 0.5% Clo (F2) and in the remaining two tanks control feed was supplemented with 0.5% TTA (F3). Four of the F1 tanks had a normal level of oxygen saturation (100% in the inlet) whereas the other four tanks had a high level of saturation (170% in the inlet). The F2 and F3 tanks all had the normal level of oxygen saturation. Throughout the experiment, the fish were fed 120% of the amount of feed needed for maximum growth (Austreng, Storebakken & Åsgård 1987), assuming a feed:weight gain ratio of 1.0.

Prior to the start of the freshwater period the salmon were exposed to continuous light (24L:0D). To induce smoltification the photoperiod was changed to 12L:12D on 2 February and back to continuous light on 24 March.

Fish and treatments in sea water

On 11 May the fish were transferred to the AKVAFORSK seawater research station at Averøy on the west coast of Norway. On arrival 60 smolts from each tank in fresh water were stocked together in each of 12 net pens (5 × 5 × 5 m), giving 720 smolts per pen representing all freshwater treatments (a total of 8840 fish). Mean body weight of the smolts was 61 g at transfer. During the seawater period (terminated 14 September) four different diets were used in a randomized block design of triplicate net pens. Seawater temperature at transfer was 9 °C increasing to about 16 °C in mid August with an average of 12 °C.

The four different seawater diets were: S1, a low fat control diet with 20% fat; S2, a high fat control diet with 29% fat; S3, the high fat control diet with

Table 1 Chemical content of feed (F1, F2 and F3) used in fresh water (experiment 1)

Type of feed	Fat (%)	Protein (%)	DM (%)	Ash (%)	NFE (%)	Additives
F1	22.8	48.1	92.1	9.4	11.8	
F2	22.7	48.8	91.3	9.4	10.4	0.5% Clo[a]
F3	24.6	48.8	93.1	9.5	10.3	0.5% TTA[a]

DM, dry matter; NFE, nitrogen free extract; TTA, tetradecylthioacetic acid.

[a]Clofibrate was obtained from Sigma Aldrich (St Louis, MO, USA). TTA was synthesized as described in Moya-Falcón *et al.* (2004).

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Table 2 Chemical content of feed (S1, S2, S3 and S4) used in sea water (experiment 1)

Type of feed	Fat (%)	Protein (%)	DM (%)	Ash (%)	NFE (%)	Additives
S1	19.8	47.2	92.4	9.3	16.2	
S2	29.2	48.0	94.7	9.9	7.6	
S3	30.1	48.4	95.3	9.9	6.8	0.5% TTA
S4	29.3	48.7	94.7	10.0	6.7	4.3% MCT[a]

DM, dry matter; NFE, nitrogen free extract; TTA, tetradecylthioacetic acid; MCT, medium chain fatty acid triacylglycerols.
[a]MCT was obtained from DeNoFa AS (Fredrikstad, Norway).

0.5% TTA added and S4, the high fat control with 14.8% of the fat substituted by MCT (Table 2).

Sampling and analyses

All diets were analysed gravimetrically for dry matter (DM) after drying at 105 °C (16–18 h), and ash (flame combustion followed by 3–4 h at 550 °C). The content of crude proteins was calculated as N × 6.25 using the semi-micro-Kjeldahl method (Kjeltec-Auto System; Tecator, Höganäs, Sweden), and crude lipid after diethyl ether extraction in a Fostec analyser (Tecator) after HCl-hydrolysis (Stoldt 1952). Proportion of nitrogen free extract (NFE) was estimated as: NFE = DM − (fat + protein + ash).

Fish were randomly sampled for analysis of fat content in the muscle at the end of the freshwater period (10 fish per tank) and after 6 weeks in sea water (60 fish per pen – five from each tank in fresh water). Plasma chloride was measured in fish sampled for analyses of muscle fat 6 weeks after sea transfer. All fish were anaesthetized (MS 222, metacaine, 0.1 g L^{-1}). Fat content in the muscle (Norwegian Quality Cut [NQC], NS 9401 1994) was analysed according to Folch, Lees & Sloana Stanley (1957) and plasma chloride was essayed using a Radiometer CMT Chloride Titrator (Copenhagen, Denmark). Plasma was separated by centrifugation of blood samples taken from the caudal vein using heparinized vacuum tubes.

Dead fish were recorded daily. Twenty-seven dead smolt from eight of the 12 pens, representing all diets, were tested for IPN by quick tests by the local veterinary service in week 9 (Nordvest Fiskehelse AS, Kristiansund, Norway), whereas seven dead fish were analysed at the end of the experiment (weeks 14–15). In addition, smolts testing positive in this screening were analysed by an immune histochemical method using anti-IPNV(sp) serum at the Norwegian Veterinary University. A natural IPN-outbreak will not always result in massive mortalities, and it should therefore be noted that the present study describes mortality and not the number of fish infected by IPNV.

Evaluation of protection from IPN

To evaluate the effect of dietary supplementation in sea water, cumulative mortalities were compared. In order to characterize this difference, the method for determination of RPS, described by Amend (1981), was used. The control group was fish fed the high fat diet without supplementation. RPS for a given dietary group was estimated by the following equation:

$$\mathrm{RPS} = 100\% \times (1 - \%\ \text{mortality in a given dietary group} / \%\ \text{mortality in the control group}).$$

Statistical analyses

All statistical analyses were performed using the SAS software package (SAS Institute Inc. 1990). Data were evaluated using one-way analyses of variance (ANOVA) or simple and multiple regression analyses. Diet in fresh water, type of vaccine, level of oxygen saturation, dietary content of fat in sea water and high fat diets in sea water were used as treatments. Experimental units were tanks for treatments in fresh water and net pens in sea water. To explain the mortality the high fat diets (S2–S4) were used in the model run for fat in muscle and plasma chloride. The proportion of the total variation explained by the model is expressed by R^2 and calculated as the marginal contribution of the mean square of the parameter (type I sum of squares). Significant differences among variables within treatments were indicated by least-square means (lsmeans) comparison. Pearson's product–moment correlations were used to describe the relationship between variables. The level of significance was chosen at $P \leq 0.05$, and the results are presented as lsmeans ± SEM.

Experiment 2

Fish and treatments

This experiment is part of a larger ongoing trial with Atlantic salmon 1+ smolts hatched in December 2004 (Marine Harvest, Slørdal, Norway). On 19 May 2006, the smolts were transferred

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Table 3 Chemical content of feed used in sea water (experiment 2)

Type of feed	Fat (%)	Protein (%)	DM (%)	Ash (%)	NFE (%)	Additives
Control	28.1	45.6	92.6	8.7	10.1	
TTA diet	27.2	47.2	94.2	9.0	10.9	0.5% TTA

DM, dry matter; NFE, nitrogen free extract; TTA, tetradecylthioacetic acid.

to the AKVAFORSK seawater research station at Averøy on the west coast of Norway. On arrival the fish were distributed in net pens with about 1100 fish in each. Mean body weight was 104 g at transfer. The part of the experiment described in this paper focuses on two different diets fed in triplicate. Seawater temperature at transfer was 9 °C, with a maximum temperature of 17 °C during the summer and an average during the 9-week period of 12 °C. The two diets were a high fat control diet and the control diet with 0.5% TTA added (Table 3). After transfer to sea water the smolts were fed the two diets until sampling for analysis of fatty acid beta-oxidation and fat content in muscle.

Sampling and analysis

Fish representing the average weight were randomly sampled for analysis of beta-oxidation in white muscle after 6 weeks at sea. The muscle samples were homogenized and the mitochondria isolated using a mitochondria isolation kit (MITO-ISO1; Sigma-Aldrich, St Louis, MO, USA). The mitochondrial fatty acid oxidation was analysed by determining the amounts of acid-soluble products in the mitochondrial fractions. The determination of acid-soluble products with [1-^{14}C] palmitoylCoA as substrate was performed as described by Lazarow (1981), except for the assay temperature, which was 20 °C.

The protein content of the mitochondrial samples was determined using a total protein kit, micro Lowry, with Peterson's modification (Lowry, Rosebrough, Farr & Randall 1951; Peterson 1977). Fat content in muscle was analysed on samples of 10 fish (NQC) from each net pen after 6 and 9 weeks of feeding, as described in experiment 1.

Statistical analyses

All statistical analyses were performed using the SAS software package (SAS Institute Inc. 1990) as described for experiment 1. Diets were used as treatments, while net pens were used as experimental units. The level of significance was chosen at $P \leq 0.05$, and the results are presented as lsmeans ± SEM.

Results

Experiment 1

IPN-mortality

In experiment 1, mortality began to increase 8 weeks after sea transfer and IPN was confirmed in all (sea water) dietary groups, both by rapid tests and immune histochemical analyses. After week 13 non-specific mortality was recorded (none of the seven dead fish sampled in weeks 14–15 tested positive for IPNV). Mean cumulative mortality from week 8 to week 13 was 5.3%.

Neither feed given in fresh water (F1 = 5.1 ± 0.3%, F2 = 5.8 ± 0.5%, F3 = 5.6 ± 1.8%), level of oxygen saturation (100% = 5.5 ± 0.4%, 170% = 4.8 ± 0.4%) nor type of vaccine (Compact 6 = 5.4 ± 0.4%, Compact 4 = 5.1 ± 0.3%) significantly affected IPN mortality in sea water. Furthermore, no statistically significant difference in mortality was observed between the groups given low-fat control diet (S1 = 4.6 ± 1.5%) compared with the groups fed high-fat control diet (S2 = 7.8 ± 0.2%), but there was a trend towards lower mortality for the low-fat groups ($P = 0.10$).

The type of high fat diet (S2–S4) given in sea water, however, had an overall significant effect ($P = 0.01$) on cumulative mortality (Fig. 1). Throughout week 8 to week 13 the dietary effect

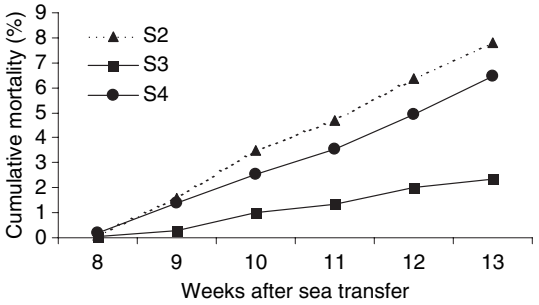

Figure 1 Cumulative IPN mortality in Atlantic salmon smolts, from week 8 to week 13 after sea transfer, fed the same level of dietary fat (experiment 1). S2 is the unsupplemented control whereas S3 and S4 are diets supplemented with TTA or MCT, respectively.

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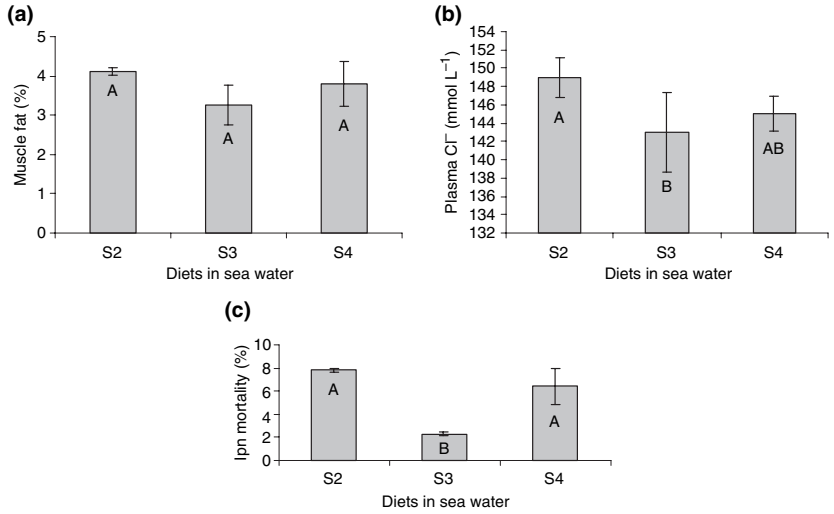

Figure 2 Fat content in the muscle (a), levels of plasma chloride (b), and mortality during a natural outbreak of IPN in weeks 8–13 after sea transfer (c) for farmed 1+ Atlantic salmon smolts fed the same dietary level of fat (29%) (experiment 1). S2 is the unsupplemented control whereas S3 and S4 are diets supplemented with TTA or MCT, respectively. Significant differences between dietary groups are indicated by different letters on the bars.

increased, explaining 20–76% of the observed variation in mortality, and the increase was significant from week 11 onwards. After 14 weeks at sea the dietary effect decreased again, coinciding with the non-specific mortality in the last period of the outbreak. Highest mortality was found in fish fed the high fat control diet (S2 = 7.8 ± 0.2%), whereas the lowest mortality was observed among those fed the high fat diet with added 0.5% TTA (S3 = 2.3 ± 0.2%). RPS for fish fed the TTA diet was 70% compared with the control group without supplementation. Dietary supplementation with TTA significantly reduced mortality compared with both the control group ($P = 0.006$) and the group supplemented with MCT (S4 = 6.4 ± 1.6%; $P = 0.02$). There was no significant difference in mortality between fish fed S2 and S4 (Fig. 2c).

Fat content in muscle

Mean fat content in the muscle of pre-smolts at the start of the freshwater period was 2.4 ± 0.1%. At the end of this period a significant difference in fat content in the muscle was observed ($P = 0.02$). Diet in fresh water was the only treatment significantly affecting the fat content. Smolts fed control diet (F1) had significantly higher fat content (3.2 ± 0.1%) than fish fed either control diet with added TTA (2.6 ± 0.3%; $P = 0.03$) or control diet with added Clo (2.5 ± 0.4%; $P = 0.02$). Overall mean fat content at sea transfer was 3.0 ± 0.4%.

After 6 weeks in sea water, just prior to the start of the IPN outbreak, no statistically significant difference in muscle fat content between smolts fed the different high fat (29%) diets was observed. However, as in fresh water, the lowest fat content was observed in the smolts fed TTA in sea water (3.3 ± 0.5%) when compared with the control groups (4.1 ± 0.1%, $P = 0.18$) (Fig. 2a).

Plasma chloride

Six weeks after sea transfer the high fat diets showed an overall effect on plasma chloride levels ($P = 0.06$). Fish fed the high fat control diet S2 had significantly ($P = 0.03$) elevated levels of chloride (149 ± 2 mmol L^{-1}) compared with fish in the TTA groups (143 ± 4 mmol L^{-1}) and non-significant but elevated levels ($P = 0.08$) compared with the MCT groups (145 ± 2 mmol L^{-1}) (Fig. 2b).

Relationship between muscle fat content, plasma chloride and IPN mortality

Positive, although not statistically significant, correlations between muscle fat content and plasma chloride ($r = 0.82$), between plasma chloride and IPN mortality ($r = 0.95$), and between muscle fat

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and IPN mortality ($r = 0.96$) were recorded. For the high fat dietary treatments identical ranking was observed for all three variables (Fig. 2a–c).

Experiment 2

Mitochondrial β-oxidation

After 6 weeks of feeding white muscle mitochondrial fatty acid β-oxidation was found to be elevated but not significantly higher ($P = 0.06$; $R^2 = 0.88$) in smolts given feed with added 0.5% TTA (30 ± 4 nmol palmitoylCoA/30 min × mg protein) when compared with smolts given the control diet (11 ± 2 nmol/30 min × mg protein) (Fig. 3a).

Fat content in muscle

As in experiment 1, the numerically lowest fat content after 6 weeks of feeding was observed in smolts fed the 0.5% TTA supplemented diet (Fig. 3b), and was significantly ($P = 0.03$, $R^2 = 0.94\%$) lower after 9 weeks (Fig. 3c).

Relationship between mitochondrial β-oxidation and fat content in muscle

The observed reduction in muscle lipid content coincided with a threefold increase in mitochondrial β-oxidation at week 6 (not measured at 9 weeks).

IPN-mortality

No natural outbreak of IPN occurred during experiment 2.

Discussion

The timing of the natural outbreak of IPN in experiment 1, 8 weeks after sea transfer, is in accordance with earlier observations made by Bowden *et al.* (2002). They found that IPN was associated with increased mortality in salmon post-smolts about 8 weeks after sea transfer, both in Norway and Shetland.

The major finding in our main experiment was the high and statistically significant reduction in IPN mortality in Atlantic salmon fed the high fat diet with added TTA (S3) in sea water, compared with the high fat diet without supplementation (S2). The experiment showed a cumulative mortality as low as 2.3% in the TTA groups compared with 7.8% in the control. This is equivalent to an RPS of 70%, which is over twice as high as that reported by the use of glucan nucleotides as dietary additives (Johnsen 2002).

Experiment 1 showed a non-significant lower IPN mortality among fish fed a diet with low fat (S1) compared with fish fed a high fat diet (S2). In previous pilot studies we have observed both negative and positive non-significant effects of increased dietary levels of fat. If no consistent effect

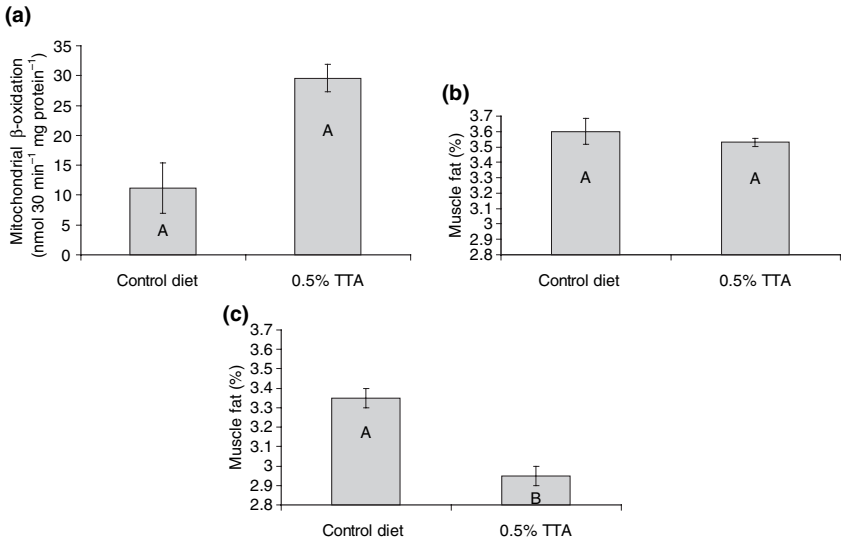

Figure 3 Fatty acid oxidation in white muscle mitochondrial fraction expressed as nmol palmitoylCoA oxidized/30 min × mg protein (a) and muscle fat content in farmed 1+ Atlantic salmon smolts fed a control diet or a diet supplemented with TTA during 6 (b) and 9 weeks (c) after sea transfer (experiment 2). Significant differences between dietary groups are indicated by different letters on the bars.

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of increase in dietary fat exists, different non-significant results in respect of levels of dietary fat would be expected by chance. One reason for the inconsistent results for different levels of dietary fat may, however, be variation between smolt groups in their capacity for fatty acid oxidation after sea transfer. If so, this is in accordance with our hypothesis that the significantly lower mortality observed in post-smolt fed high fat diets with added TTA (S3) in experiment 1 is a consequence of higher capacity for energy utilization and thus enhanced access to energy to combat osmotic imbalance during the first weeks in sea water. In support of this, the activity of palmitoyl-CoA oxidation in mitochondria isolated from white muscle was, in experiment 2, found to be increased by three times after 6 weeks of feeding TTA when compared with controls, indicating increased fatty acid oxidizing capacity in the smolts.

In the freshwater period addition of both TTA and Clo led to reduced body lipid stores compared with groups given the control feed. During the first 6 weeks in sea water, however, a similar but not significant ($P = 0.17$) reduction in muscle fat was observed with TTA feeding, compared with smolts of identical body weight fed the high fat control diet. In experiment 2, smolts fed the diet containing TTA also had lower fat in muscle than the control groups (significant after 9 weeks), supporting the previous observation. Hence, the observation of TTA-induced fatty acid oxidation activity in white muscle mitochondria suggests a re-allocation of dietary fatty acids from storage to energy producing oxidation in the TTA-fed smolts.

Moya-Falcón *et al.* (2004) showed that administration of TTA to Atlantic salmon over an 8-week period in sea water led to accumulation of sulphur oxygenated TTA metabolites in the kidney. Metabolites in the kidney were not analysed in the present study, however, levels of magnesium in plasma and haematocrit in the blood 4 months after sea transfer showed no significant difference between smolts fed high fat diets with or without supplementation of TTA (results not presented). Further evaluation of the significance of this potentially negative effect of TTA in fish is necessary.

It is well known that marine teleosts have developed a strategy for seawater adaptation, involving drinking of water and excretion of salts in order to compensate for water losses (Conte 1969). The rate of drinking varies with species, water salinity and temperature. For salmonids, the drinking rate varies from 40 to 130 mL kg^{-1} day^{-1} (Parry 1966; Shehadeh & Gordon 1969; Potts, Foxster & Stather 1970). Osmoregulatory imbalance and hence reduced seawater tolerance after sea transfer may be seen as increased levels of plasma chloride in the smolts (Staurnes, Sigholt, Åsgård & Baeverfjord 2001). Observations of significantly elevated plasma chloride levels in smolts fed the high fat control diet S2 (149 mmol L^{-1}) when compared with the normal level in fish in the TTA groups (143 mmol L^{-1}) may thus suggest reduced physiological stress in the latter groups. As small fish have a higher gill surface area and a greater body surface area to mass ratio than larger fish (McDonald & Milligan 1997), small fish like smolts will probably experience high osmoregulatory disturbances during seawater acclimatization. Increased capacity for fat utilization and hence increased energy access may explain the observation of significantly lower levels of plasma chloride and reduced osmotic stress in smolt fed TTA compared with the controls 6 weeks after sea transfer, 2 weeks prior to the outbreak of disease. Addition of TTA during the critical and temporary high metabolic energy-requiring period of 1^+ smolts after sea transfer thus seems to increase their ability to resist IPNV reactivation or infection.

Smolt fed the high fat diet containing MCT (S4) also showed somewhat reduced IPN mortality ($P = 0.08$) compared with the high fat control groups. The cumulative mortality was 6.4% for smolts fed S4. This is equivalent to an RPS of 18%. Some reduction in body lipid stores 6 weeks after sea transfer was indicated for the TTA-treated smolts. As discussed above, reduction in body lipid may suggest that more of the available dietary lipid energy has been routed towards oxidation. Supplying the smolts with more easily oxidizable medium chain fatty acids thus seems to be one possible method of reducing the osmotic stress encountered by the salmon smolts during the first weeks after seawater transfer. Results from previous studies using MCT in diets for salmon may suggest that a higher inclusion than used in the present study is possible (Røsjø *et al.* 2000).

During the natural outbreak of IPN in the present study no effect of the IPN vaccine alone or with dietary interactions was observed. These observations are in agreement with the low percentage of Norwegian salmon farmers (12%) that consider the IPN vaccine as an effective measure to reduce outbreaks (FHL & VESO 2003). The

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apparent contradiction between the good results obtained in controlled challenge tests by the vaccine companies and the rather uncertain efficacy observed in commercial post-smolts is a great problem for the farming industry. A possible explanation might be that due to stimulation of non-specific immunological defence mechanisms by certain components in the vaccine, highly effective oil-emulsified vaccines mask possible differences in disease susceptibility at low and moderate mortalities. In line with this, among post-smolts vaccinated with an oil-emulsified injected vaccine protective against *Aeromonas salmonicida* and *Vibrio salmonicida*, Eggset *et al.* (1997) observed 18.8% IPN mortality in the unvaccinated fish, compared with 0–4% in the vaccinated groups. However, as all smolts used in commercial salmon farming in Norway are normally vaccinated, alternative strategies to reduce IPN mortality are even more important.

The present study demonstrates that whereas vaccination still seems to be far from solving the IPN problem in salmon farming, dietary addition of TTA significantly increased survival (RPS = 70%) during a natural outbreak of IPN after sea transfer of 1^+ salmon. It is suggested that TTA results in a re-allocation of dietary fatty acids from storage to energy producing oxidation due to increased mitochondrial beta-oxidation capacity in white muscle. The increased access to energy may explain the observation of significantly lower levels of plasma chloride and hence reduced osmotic stress in smolt fed TTA compared with controls 6 weeks after sea transfer, 2 weeks prior to the outbreak of the disease. During this critical, but temporary, high metabolic energy-requiring period of 1^+ smolts after sea transfer, dietary addition of TTA seem to increase the ability of salmon smolt to resist IPN. For an optimal use of TTA in commercial salmon farming more information concerning doses and treatment periods and the metabolic effects of TTA are needed.

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Paper IV

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Increased survival by feeding tetradecylthioacetic acid during a natural outbreak of heart and skeletal muscle inflammation in S0 Atlantic salmon, *Salmo salar* L.

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Abstract

We have previously documented increased survival by feeding tetradecylthioacetic acid (TTA) during a natural outbreak of infectious pancreatic necrosis in post-smolt S1 Atlantic salmon. The aim of the present study was to test the effects of dietary TTA in S0 smolt at a location where fish often experience natural outbreaks of heart and skeletal muscle inflammation (HSMI) during their first spring at sea. The experimental groups were fed a diet supplemented with 0.25% TTA for a 6-week period prior to a natural outbreak of HSMI in May 2007. Relative percent survival for the groups fed TTA was 45% compared with control diets, reducing mortality from 4.7% to 2.5%. Expression of genes related to lipid oxidation was higher in cardiac ventricles from salmon fed TTA compared with controls. In addition, salmon fed TTA had periodically reduced levels of plasma urea, and increased cardiosomatic index and growth. Reduced mortality and increased growth after administration of TTA may be related to a combination of anti-inflammatory effects, and an altered metabolic balance with better protein conservation because of increased lipid degradation.

Keywords: Atlantic salmon, heart and skeletal muscle inflammation, lipid metabolism, peroxisome proliferator activated receptors, tetradecylthioacetic acid, urea.

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Introduction

Heart and skeletal muscle inflammation (HSMI) was first reported in farmed salmon along the mid-Norwegian coast in 1999, and this area still has the highest number of outbreaks in Norway, even though the disease is now found along the entire coast (Olsen, Bornø, Colquhoun, Flesjå, Haldorsen, Mo, Nilsen, Skjelstad & Hjeltnes 2007). A disease outbreak resembling HSMI was also reported from Scotland (Ferguson, Kongtorp, Taksdal, Graham & Falk 2005). The incidence of HSMI in Norway continues to increase. In 2006 at least 94 locations were affected by the disease, compared with 83 in 2005 and 54 in 2004 (Olsen *et al.* 2007). Severe and repeated outbreaks of HSMI result in high economic losses because of lowered appetite and high mortality, and even though mortality and duration of an outbreak can be variable, the morbidity may be very high (Kongtorp, Taksdal & Lyngøy 2004a; Watanabe, Karlsen, Devold, Isdal, Litlabø & Nylund 2006).

Heart and skeletal muscle inflammation is suggested to be a viral infection (Kongtorp *et al.* 2004a) that affects salmon during the marine stage. The disease was originally described in salmon 5–9 months post-sea transfer in Norway (Kongtorp, Kjerstad, Taksdal, Guttvik & Falk 2004b; Kongtorp *et al.* 2004a; Watanabe *et al.* 2006; McLoughlin & Graham 2007). Outbreaks now seem to occur during the whole year, but most frequently during spring (May–June) and autumn (October–November) (Olsen *et al.* 2007).

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Affected fish may become anorexic and display abnormal swimming (Kongtorp *et al.* 2004a), but may also be slow moving without any other external signs of disease (Watanabe *et al.* 2006). HSMI is reported to cause histopathological lesions in heart and skeletal muscle. The lesions associated with HSMI are partly similar to those described for two other serious diseases in intensive salmon farming; pancreas disease (PD) and cardiomyopathy syndrome (CMS), making it difficult to distinguish between them (Kongtorp *et al.* 2004a,b). The main difference between HSMI and PD is the absence of pancreatic lesions in HSMI (reviewed by McLoughlin & Graham 2007).

No vaccines or practical treatments exist for avoiding or reducing the extent of HSMI outbreaks in salmon farming. As far as we are aware, the present study is the first describing nutritional effects on HSMI mortality.

Tetradecylthioacetic acid (TTA) is a saturated fatty acid where the third methylene group from the carboxylic end of the chain is replaced by a sulphur atom (Muna, Bolann, Chen, Songstad & Berge 2000). TTA has been reported to increase the capacity for beta-oxidation in rats (Asiedu, Skorve, Willumsen, Demoz & Berge 1993), and in white muscle (Rørvik, Alne, Gaarder, Ruyter, Måseide, Jakobsen, Berge, Sigholt & Thomassen 2007) and liver (Moya-Falcón, Hvattum, Dyrøy, Skorve, Stefansson, Thomassen, Jakobsen, Berge & Ruyter 2004) in Atlantic salmon, *Salmo salar* L. L-carnitine plays an important role in transporting long-chain fatty acids across the inner mitochondrial membrane to the site of oxidation and facilitates removal of short-chain organic acids from mitochondria (reviewed by Harpaz 2005). Thereby, intramitochondrial coenzyme A becomes available to participate in the beta-oxidation pathway.

Compared with salmon transferred to sea in spring 1-year post-hatch (S1), salmon transferred to sea during autumn after only 6 months in fresh water (S0) are reported to experience a period of reduced performance during their first spring at sea. This period coincides with the time of most frequent HSMI outbreaks. As TTA was earlier found to have a significant effect on mortality caused by a natural outbreak of infectious pancreatic necrosis (IPN) (Rørvik *et al.* 2007), we evaluated how salmon transferred to sea as S0 smolts responded to a dietary supplementation of TTA during spring after one sea-winter. In this study, the dietary effects of TTA during a natural outbreak of HSMI including parameters such as feed intake, growth rate, feed efficiency, condition factor and muscle fat were described.

Materials and methods

Fish and treatments

The present study was conducted at the NOFIMA research station on the Norwegian west coast (Averøy), using Atlantic salmon (S0) transferred to sea in November 2006. In mid-March (20th) 2007, 550 fish were stocked into each of eight net pens ($5 \times 5 \times 5$ m). A randomized block design was used, with all the net pens located on the same pier. Initial mean body weight was 221 ± 1 g and was 1585 ± 62 g at the final sampling in September. Four different diets (Table 1) were fed during the first 6 weeks (period 1). The basic diets (Table 2) were produced by Biomar AS. TTA was obtained from Thia Medica, and carnitine from Lohman Animal Health. The experimental diets were prepared by coating the basic diet in a blender. Carnitine was dissolved in distilled water and coated onto the surface of the feed pellets for diet E2, to a final inclusion of 0.2%. A similar amount of water was coated onto the two other high fat diets (C2 and E1) and all three diets were dried at room temperature. C2 was then coated with 2% rapeseed oil and E1 and E2 were coated with TTA dissolved in the same amount of rapeseed oil, to a final level of 0.25%. Finally, 1% of capelin oil was

Table 1 Experimental diets used in this study

Diet code	Diets
C1	Basic CPK-diet
C2	C1 + 2% rapeseed oil and 1% capelin oil
E1	C2 + 0.25% TTA
E2	E1 + 0.2% carnitine

TTA, tetradecylthioacetic acid.

Table 2 Chemical content of the basic diets used during the experimental periods

	Basic diet period 1 CPK 200	Basic diet period 2 CPK 500
Dry matter (%)	93.2	94.5
Protein (%)	42.7	40.2
Lipid (%)	29.6	27.7
Ash (%)	8.5	7.8
NFE (%)	12.4	18.8

NFE, nitrogen-free extracts.

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coated onto the three diets. From week 7 onwards (period 2), the fish were fed the same diet as in feeding period 1, but without added TTA. In period 2, the commercial basic diet was adjusted to a bigger fish size in accordance with the manufacturer's guidelines and the pellet size was increased (CPK 500; Table 2). All the diets were fed in excess in duplicated net pens, and waste feed sampling was performed during both feeding periods.

Sampling and analyses

The basic diets were analysed gravimetrically for dry matter (DM) after drying at 105 °C (16-18 h), and for ash (flame combustion followed by 3-4 h at 550 °C). The content of crude proteins was calculated as N × 6.25 using the semi-micro-Kjeldahl method (Kjeltec-Auto System; Tecator), and crude lipid after diethyl ether extraction in a Soxtec analyser (Tecator) after HCl-hydrolysis (Stoldt 1952). The proportion of nitrogen-free extract (NFE) was estimated as: NFE = DM − (fat + protein + ash).

Thirty fish were randomly sampled at the start of the experiment. Thereafter 10 fish from each cage, representing the average weight, were sampled every third week until August (2007). Individual body weights (to the nearest g) and heart weights (to the nearest mg) were recorded for sampled fish. All sampled fish were anaesthetized (MS 222 metacaine, 0.1 g L^{-1}; Alpharma Animal Health Ltd) and killed by a blow to the head.

During the disease outbreak dead fish were recorded daily. The local veterinary service (Nordvest Fiskehelse AS, Kristiansund, Norway) was contacted immediately when mortality increased and a disease outbreak was suspected. The veterinarians dissected organs from 11 fish from five of the eight pens, representing all dietary treatments. In addition, two fish from the cage from which the fish originated were analysed. Samples were examined by the National Veterinary Institute of Norway (NVI, Oslo) using histological analysis. Myocarditis was observed in all fish, but since pancreas was not included in the two first fish groups submitted, a final diagnosis of HSMI was only made in the last group submitted. The diagnosis was then confirmed in fish on all diets except for the one only with TTA (E1). A natural disease outbreak, such as the present case of HSMI, will not always result in massive mortalities, and it should therefore be noted that the present paper describes mortality and not the number of fish infected by HSMI.

Every sixth week blood samples were taken from the caudal vein using ethylenediaminetetraacetic acid vacuum tubes, from nine fish per cage. Plasma was obtained by centrifugation at 4 °C and 630 ***g*** for 10 min. Equal volumes of plasma from three fish were pooled in each of three cryotubes, frozen in liquid nitrogen and then stored at −80 °C for later analyses. Urea was analysed using a Biochrom 30 ion-exchange amino acid analyser (Biochrom Ltd), equipped with a thermostatted autosampler, ninhdydrin post-column derivatization and photometric detection at 440 and 570 nm. Prior to analysis, samples were deproteinized (50 mg sulphosalicylic acid mL^{-1} of plasma) and filtered (0.22 μm Ultrafree CL; Millipore).

The cardiac ventricle from fish fed the high-fat control diet (C2) and the TTA-supplemented diet (E1) were sampled every sixth week for analysis of gene expression, with focus on genes known to be involved in fatty acid oxidation. At each sampling, total RNA was extracted from the cardiac ventricles of six fish from each net pen (72 fish in total for all the samplings) using TRIzol™ reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. The integrity of the isolated total RNA was verified by ethidium bromide-stained agarose gels and by measuring the ratio (260/280) of the samples. To remove traces of genomic DNA from the samples 10 μg of RNA were subjected to DNase-treatment using a Turbo DNA-Free Kit (Ambion). After DNase inactivation and RNA quantification, 1.2 μg of RNA were reverse transcribed using oligo $d(T)_{16}$ primer and the TaqMan® Reverse Transcription Reagent Kit (Applied Biosystems). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed by use of the LightCycler®480 (Roche) and gene specific primers (Table 3). For the real-time RT-PCR reaction 2× SYBR Green I Master Mix (Roche), 0.4–0.5 nM of each primer and the cDNA template were mixed in a reaction volume of 12 μL. PCR amplifications were performed in triplicate. The amplification efficiencies of all examined amplicons were calculated from a serial dilution of a cDNA pool using LightCycler© Software Version 1.2.0169. A three step PCR was run for 45 cycles (15 s at 95 °C; 15 s at 58 °C; 15 s at 72 °C) with a preincubation of 5 min at 95 °C for activating the FastStart Taq DNA polymerase and for a melting curve analysis at the end of the program to verify specific amplification.

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Table 3 Primers for quantitative real-time polymerase chain reaction used in this study

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	GenBank accession no.
EF1α	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC	AF321836
PPARα	TCCTGGTGGCCTACGGATC	CGTTGAATTTCATGGCGAACT	DQ294237
PPARβ	GAGACGGTCAGGGAGCTCAC	CCAGCAACCCGTCCTTGTT	AJ416953
PPARγ	CATTGTCAGCCTGTCCAGAC	TTGCAGCCCTCACAGACATG	AJ416951
ACO	CCTTCATTGTACCTCTCCGCA	CATTTCAACCTCATCAAAGCCAA	DQ364432
LPL	TGCTGGTAGCGGAGAAAGACAT	CTGACCACCAGGAAGACACCAT	BI468076
CPT-1	TCCCACATCATCCCCTTCAACT	TGTCCCTGAAGTGAGCCAGCT	AM230810

EF1α, elongase factor 1 alpha; *PPARα,β,γ*, peroxisome proliferator-activated receptor alpha, beta, gamma; *ACO*, acylCoA oxidase; *LPL*, lipoprotein lipase; *CPT-1*, carnitine palmitoyl transferase 1.

Calculations

Weight gain was calculated as thermal growth coefficient (TGC) (Cho 1992): TGC = $(W_b^{1/3} - W_a^{1/3}) \times (\sum T)^{-1}$, where W_b is the final weight, W_a is the initial weight and $\sum T$ is the sum of day degrees during the period. To simplify the figures, the TGC is multiplied by 1000. Feed conversion ratio (FCR) was calculated as (kg feed eaten) × (kg final biomass − kg initial biomass + kg dead fish)$^{-1}$. Cardiosomatic index (CSI) was calculated as ventricle weight (g) × [body weight (g)]$^{-1}$ × 100. Relative percent survival (RPS) was calculated as described by Amend (1981): 100% × (1 − % mortality in the experimental group/% mortality in the control group).

Statistical analyses

If not otherwise stated, all statistical analyses were performed using the SAS software package (SAS Institute Inc. 1990). Data were analysed using analyses of variance (ANOVA) or simple and multiple regression analyses. The model used mortality, CSI, FCR, TGC or urea concentration in plasma as dependent variables, and block, side of the pier, sampling point and dietary treatment as class variables. Experimental units were net pens. The proportion of the total variation explained by the model is expressed by R^2 and calculated as the marginal contribution of the mean square of the parameter (type III sum of squares). Significant differences among variables within treatments were indicated by least-square means (lsmeans) comparison. The level of significance was chosen at $P \leq 0.05$, and the results are presented as lsmeans ± SEM, if not otherwise stated.

The relative gene expression was analysed using the Relative Expression Software Tool (REST-384© version-2; Pfaffl, Horgan & Dempfle 2002). The expression levels of the target genes were normalized to the expression level of the housekeeping gene *EF1α*, which was verified to be unaffected by the dietary treatments using the REST-384 software tool. Significant differences in gene expression between the high-fat control diet (C2) and the diet supplemented with TTA (E1) at each sampling point were calculated by the Pair Wise Fixed Reallocation Randomization Test© (2000 randomizations). Mean values of the triplicates were used for each of the cardiac ventricles ($n = 12$) representing each treatment in the REST-384 software.

Results

In late May, mortality started to increase and HSMI was confirmed to be the cause of death. At the beginning of June the mortality levelled off (Fig. 1). As no differences were observed between the two control diets or between the experimental diets with added TTA, the diets were pooled into one control and one TTA group. Both the side of the pier and diet was found to significantly influence total mortality, together explaining 81% of the observed variation in the model. Supplementing diets with TTA was found to significantly reduce total mortality during the HSMI outbreak, as shown in Fig. 2. The results show an RPS between the TTA-group and the control diets of 45%.

Body weight at the end of the experimental period was influenced by both block ($P < 0.001$) and diet ($P = 0.04$). TTA diets resulted in a higher final body weight (1606 ± 29 g) than control diets (1580 ± 31 g). For both control and TTA-fed fish the TGC was lowest and feed conversion ratio (FCR) was highest between weeks 6 and 12 after the start of the experiment. During this period fish fed TTA-supplemented diets had significantly ($P = 0.04$) higher TGC, and a non-significant improvement of feed conversion ($P = 0.23$; Fig. 3).

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Except during this period, which coincided with the disease outbreak, no differences in growth between the two groups were observed.

As a result of individual variations in the CSI, we used the mean value of the three samplings performed after the outbreak (in June and July). Both diet ($P = 0.05$), block ($P = 0.008$) and side of the pier ($P = 0.04$) were found to significantly influence CSI, together explaining 91% of the observed variation. CSI was significantly higher in fish fed a TTA-supplemented diet (0.137 ± 0.004) compared with control fish (0.132 ± 0.003). A regression analysis of mortality revealed a significant effect of CSI ($P = 0.003$) and block ($P = 0.017$), together explaining 86% of the observed variation ($P = 0.007$, $R^2 = 0.86$).

In June (week 25) urea concentration in blood plasma was found to be significantly decreased by dietary treatment (TTA vs. control: $P = 0.004$, $R^2 = 0.95$; Fig. 4), 6 weeks after the end of feeding TTA. This sampling was performed at the end of the disease outbreak. No significant effect of diet or dietary level of fat on plasma urea concentration was observed at the sampling in May, at termination of the TTA-feeding period.

No statistically significant differences in cardiac ventricle gene expression between dietary groups were observed at the sampling in May (week 19). However, 6 weeks after the end of TTA-feeding (week 25), the expression of the lipid metabolic genes, *peroxisome proliferator activated receptors* (*PPAR*)-α and -β, *peroxisomal acylCoA oxidase* (*ACO*) and *lipoprotein lipase* (*LPL*) was significantly up-regulated (2–3 times higher) in fish fed TTA (Fig. 5). The mitochondrial transporter *carnitine palmitoyl transferase I* (*CPT I*) showed a similar but non-significant up-regulation. The June sampling was performed at the time when mortality caused by HSMI was ending. In September (week 39) the gene expressions were no longer significantly different, but a trend of down-regulated *PPAR*β mRNA transcription ($P = 0.08$) was observed for the TTA fed fish. The ratio between *PPAR*β and

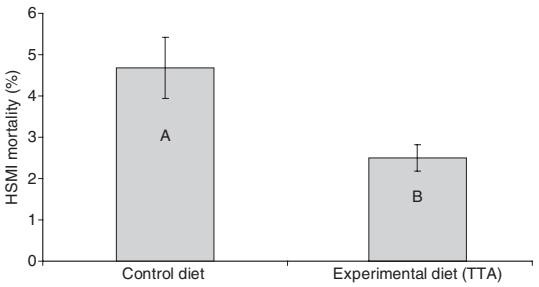

Figure 2 Total mortality during a natural heart and skeletal muscle inflammation outbreak in spring for control and experimental groups of farmed S0 Atlantic salmon. Significant differences between dietary groups are indicated by different letters on the bars.

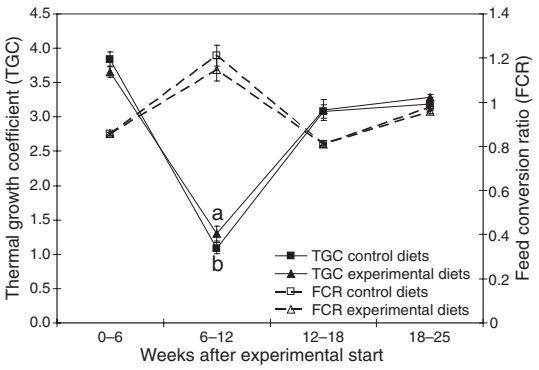

Figure 3 Changes in thermal growth coefficient and feed conversion ratio for S0 Atlantic salmon fed control or experimental diets throughout the experimental period.

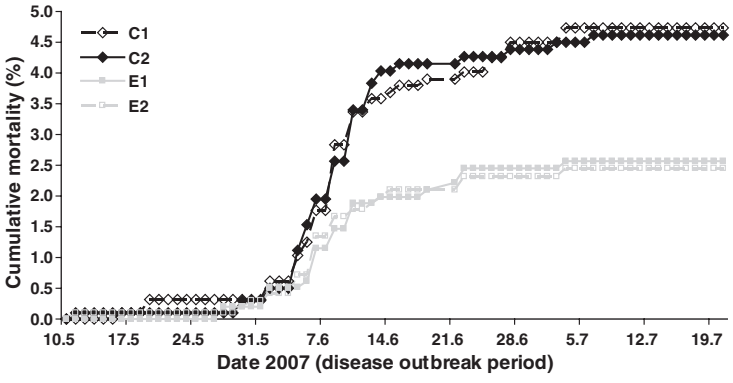

Figure 1 Cumulative mortality during a natural outbreak of heart and skeletal muscle inflammation in May–July (period 2) in farmed S0 Atlantic salmon given four different diets during period 1, either a low-fat control diet (C1), high-fat control diet (C2), C2 with added 0.25% tetradecylthioacetic acid (E1) or E1 with added 0.2% carnitine (E2).

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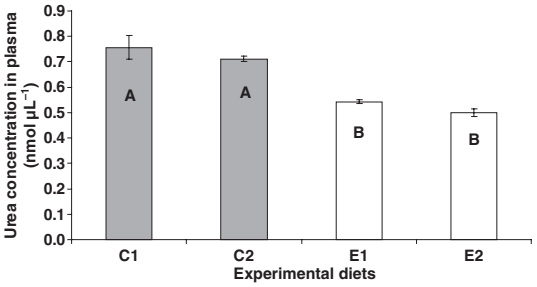

Figure 4 Urea concentration in blood plasma of S0 Atlantic salmon in June (week 25) after a natural outbreak of heart and skeletal muscle inflammation. The fish were fed four different diets in period 1: C1 and C2 are control diets with different fat levels, E1 and E2 are two diets with added tetradecylthioacetic acid (Table 1). Significant differences between dietary groups are indicated by different letters on the bars.

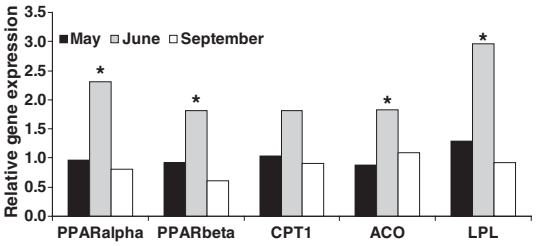

Figure 5 Relative expression compared with controls of genes involved in lipid metabolism in cardiac ventricles of Atlantic salmon given a diet supplemented with 0.25% tetradecylthioacetic acid for 6 weeks (March–May). *Above bar denotes significant differences between dietary treatments, $P < 0.05$, $n = 2$. *PPARα,β*, peroxisome proliferator-activated receptor alpha and beta; *CPT I*, carnitine palmitoyltransferase I; *ACO*, acyl-CoA oxidase; *LPL*, lipoprotein lipase.

PPARα mRNA expression was about 0.4 in the May and September samplings, while in the June sampling the ratio was close to 1. *PPARγ* was hardly detectable in the ventricles at any sampling point (results not shown).

Discussion

This is the first report showing a positive nutritional effect on HSMI mortality. The main finding was the increased survival during an HSMI outbreak of salmon fed a TTA-supplemented diet. In addition, fish fed TTA had an increased CSI, lowered plasma urea content and higher expression of genes relevant for fatty acid oxidation in the heart.

The timing of the natural outbreak of HSMI, in May and June, is as reported by Kongtorp, Halse, Taksdal & Falk (2006) and Watanabe *et al.* (2006). Kongtorp *et al.* (2006) studied a commercial farm with a history of recurrent outbreaks of HSMI, and first diagnosed fish with HSMI during May followed by a clinical outbreak of HSMI in June. The present outbreak of HSMI also coincided with outbreaks in several commercial farms along the Norwegian coast, and Olsen *et al.* (2007) reports May–June and October–November to be the periods with the highest number of diagnoses of HSMI.

Mortality started at different times in different net pens, and was highest at one side of the pier. This may indicate that the disease spread among the cages and also that the cages experienced different infection pressure during the outbreak. A block design was used in our experiment, enabling us to document isolated dietary effects. Our study was not designed to differentiate and measure infection pressure, but the results support the idea that HSMI is contagious and spreads from cage to cage (Kongtorp *et al.* 2004a,b).

Myocarditis was observed in all sampled fish submitted to NVI during the disease outbreak. This lesion is present in fish affected by HSMI, but is also reported for other diseases such as PD (Ferguson, Roberts, Richards, Collins & Rice 1986) and CMS (Ferguson, Poppe & Speare 1990). Together, these three diseases cause significant losses for the salmon farming industry, and thus far no reliable methods for avoiding and combating them are available. For PD some nutritional studies have been performed, showing an effect of additional antioxidants such as vitamin E on mortality rates (Raynard, McVicar, Bell, Youngson, Knox & Fraser 1991; McCoy, McLoughlin, Rice & Kennedy 1994). However, the present study is the first to show any dietary effect on mortality caused by HSMI. TTA is an antioxidant (Muna, Doudin, Songstad, Ulvik & Berge 1997) and is suggested to exert anti-inflammatory effects in mice and humans (Fredriksen, Ueland, Dyrøy, Halvorsen, Melby, Melbye, Skalhegg, Bohov, Skorve, Berge, Aukrust & Frøyland 2004; Dyrøy, Yndestad, Ueland, Halvorsen, Damås, Aukrust & Berge 2005) This may thus be a contributing factor to the reduced mortality observed for TTA-fed fish in the present study.

The CSI was found to be higher in fish fed the TTA-supplemented diets compared with controls. In addition, a similar increase was observed in the hepatosomatic index for salmon fed the TTA-supplemented diets, however this was not

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significant (results not shown). TTA is known as a peroxisome proliferator (reviewed by Bremer 2001), increasing both number and size of the peroxisomes and mitochondria in several organs, as shown in studies with rats (Berge, Aarsland, Kryvi, Bremer & Aarsaether 1989). This may in turn lead to a bigger heart and liver and also to a higher capacity for fatty acid β-oxidation, as we have recently observed in white muscle of S1 Atlantic salmon (Rørvik *et al.* 2007). In agreement with this, the expression of *PPAR* α and β, as well as mitochondrial *CPT I* and peroxisomal *ACO*, were up-regulated in the cardiac ventricle in fish fed TTA. The expression profiles of PPARs in different tissues, including heart, have been described in two marine species (Leaver, Boukouvala, Antonopoulou, Diez, Favre-Krey, Ezaz, Tocher, Batista & Krey 2005). In Atlantic salmon expression of PPARs has been studied in liver and skeletal muscle (Kleveland, Ruyter, Vegusdal, Sundvold, Berge & Gjøen 2006), but to the best of our knowledge this is the first report in hearts of Atlantic salmon. PPARα in animals and humans is highly expressed in tissues with significant fatty acid catabolism, including the heart (reviewed by Zandbergen & Plutzky 2007). PPARβ is more ubiquitously distributed, but has recently been suggested to be of significant importance in regulation of metabolic energy balance in muscle tissue (Grimaldi 2007).

In our previous study (Rørvik *et al.* 2007) describing the significant reduction of IPN mortality with dietary TTA treatment, we suggested this effect, at least in part, to be connected to more available metabolic energy because of increased mitochondrial β-oxidation in white muscle. To what extent these effects of TTA may be involved in the significantly reduced HSMI mortality reported here, remains to be elucidated.

During the HSMI outbreak, fish fed TTA had a higher growth rate and a lower FCR, which may be as a result of their overall better health. A high demand for metabolic energy in this period may be met by the higher β-oxidation capacity of liver (Moya-Falcón *et al.* 2004) and white muscle (Rørvik *et al.* 2007) of Atlantic salmon fed TTA. In mammals, it has been proposed that PPARα decreases mRNA expression of several enzymes involved in amino acid metabolism, based on results of comparison between PPARα-null mice with wild-type mice (Kersten, Mandard, Escher, Gonzalez, Tafuri, Desvergne & Wahli 2001). Arginase, the enzyme that catalyses the conversion of arginine into urea and ornithine, was found to be highest in PPARα-null mice compared with controls, although a synthetic PPARα ligand did not induce the same response (Kersten *et al.* 2001). In the present study, at the time when PPARα was significantly upregulated in hearts from salmon that had been fed TTA-supplemented diets, urea levels in plasma were significantly reduced. The reaction to TTA and elevated PPARα in fish may therefore include down-regulation of arginase expression and arginine catabolism, as in mammals.

Administration of dietary-supplemented TTA has earlier been found to result in changes in kidney morphology (Gjøen, Kleveland, Moya-Falcòn, Frøystad, Vegusdal, Hvattum, Berge & Ruyter 2007) and accumulation of sulphur-oxygenated TTA metabolites in the kidneys (Moya-Falcón *et al.* 2004). In the present study, no gross changes in the kidneys of fish fed TTA were observed. This may be as a result of the level of dietary TTA, duration of supplementation and season/timing of feeding.

In conclusion, the reduced HSMI mortality observed in Atlantic salmon may be as a result of a combination of anti-inflammatory effects and an altered balance between fatty and amino acid metabolism. Although more studies are required to understand in detail the mechanisms involved, the present results suggest the importance of developing 'functional feeds' in fish farming.

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