Norwegian University of Life Sciences
Faculty of Biosciences

Philosophiae Doctor (PhD)
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Tetradecylthioacetic acid (TTA) – A functional feed ingredient for Atlantic salmon affecting early sexual maturation, cardiac robustness and β-oxidative capacity

Tetradecylthioacetic acid (TTA) – en funksjonell fôringrediens som påvirker tidlig kjønnsmodning, hjerte robusthet og β-oksidativ kapasitet hos Atlantisk laks

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Tórshavn, March 2018

Regin Arge

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Abbreviations

S0	Smolt transferred to sea less than one year post hatching
S1	Smolt transferred to sea more than one year post hatching
ACO	Acyl-CoA oxidase
CPT1	Carnitine palmitoyltransferase 1
CSI	Cardio somatic index (heart W(g) / body W (g) * 100)
GSI	Gonado somatic index (gonad W(g) / body W (g) * 100)
PPAR	Peroxisome proliferator activated receptor
TTA	Tetradecylthioacetetic acid
FAO	Fatty acid oxidation
ASP	Acid soluble products
AMPK	5-AMP-activated protein kinase

Summary

Utilisation of fatty acids highly dominates energy metabolism in high performance fish as the Atlantic salmon. It is well established knowledge that feed intake and growth of salmon is highly influenced by water temperature and day length. Another significant factor influencing growth and fat deposition in Atlantic salmon, is the initiation of puberty and the development in sexual maturation. Previous research has shown that maturation in salmon is a complex process depending on different stimuli gained from both external factors like photoperiod and internal factors like age and state of energy reserves. After the maturation process has been initiated during early winter, it has been shown that the availability of appropriate energy reserves during the spring period is a major factor affecting continuance of the maturation process, and low energy or fat levels may arrest further progress. Female salmon are known to invest more energy in the development of gonads than male salmon, hence, females normally mature later in life than males, most often in the autumn after two or more sea winters. The combination of these factors significantly influences the production biology of farmed Atlantic salmon and is the scope of this thesis.

In this thesis tetradecylthioacetic acid (TTA) is strategically used in the test diet to alter the fat accumulation in farmed S0 Atlantic salmon muscle during their first spring at sea (*paper I*). It is shown that lower fat accumulation in spring leads to reduced incidence of early maturation in male grilse in the succeeding autumn by 1/3 compared to the control group receiving the same feed without TTA supplementation. To describe the decrease of TTA in fish muscle after termination of TTA supplementation in the feed, an elimination model is further developed in this study (*paper I*). As salmon females normally mature at later life stage compared to males, another experiment with S1 salmon was conducted to investigate possible different responses between the sexes regarding TTA treatments (*paper II*). It is shown that muscle fat in males and females differed significantly as a response to dietary TTA. The muscle fat content during the first spring was significantly lower in females compared to males. In contrast, during the second spring, fat content was significantly more reduced in males than in females. Condition factor is shown to follow a similar pattern as muscle fat. The results in paper II indicate that the difference in male and female fat accumulation dynamics is related to sex-specific reproduction biology of Atlantic salmon.

To obtain more insight into the response to TTA treatments, a study of the salmon heart was selected due to its characteristics as a highly energy consuming vital organ (*paper III*). It is shown that fish given TTA supplemented feed had a smaller decrease in heart weight relative to fish bodyweight (CSI) in a period after sea transfer compared to the control. This coincided with lowered condition factor and muscle fat in the treated fish. To examine this further, isolated salmon heart cells were held in culture and pre-stimulated with TTA in order to increase the endogenous concentration of this bioactive component. Radiolabelled fatty acid (FA) was supplemented to culture media to study the effects of endogenous TTA on uptake, incorporation in lipid classes and β-oxidation. It is shown that heart cells receiving 120µM TTA had higher uptake of radiolabelled FA and formation of the β-oxidation products CO_2 and other acid-soluble products. The molecular mechanisms underlying this were studied in an additional experiment. Salmon held in tanks on land were subjected to injections of TTA in increasing doses. By using gene expression (real-time quantitative RT-PCR) analyses, it is shown that genes regulating cell growth, peroxisomal FA oxidation, FA elongation and desaturation, were upregulated in the heart of TTA treated fish. In contrast, genes involved in FA transport into the mitochondria were not influenced. Taken together, the findings in paper III show that TTA treatments lead to increased heart size, possibly by increasing the expression of genes regulating heart cell growth and enhanced energy production by stimulation of FA oxidation.

In conclusion, the findings in this thesis demonstrate that TTA in defined doses, without compromising fish growth, may successfully be used strategically during energy demanding periods for the fish. Additionally, TTA reduces incidence of early sexual maturation, increases cardiac robustness and oxidative capacity in farmed salmon. Further, the effect of TTA may be influenced by the sex and size of the fish.

Implementation of the knowledge generated by the work in this thesis has the potential of being beneficial for the salmon farming industry.

Sammendrag

Utnyttelse av fettsyrer dominerer energimetabolismen i høytytende fisk som atlantisk laks. Det er kjent at fôrinntak og vekst hos oppdrettslaks i høy grad påvirkes av vanntemperatur og daglengde. Men en annen viktig faktor som påvirker vekst og deponering av fett hos laks, er begynnelsen av puberteten og den videre utvikling av kjønnsmodningen. Tidligere studier har vist at kjønnsmodning hos laks er en kompleks prosess, som avhenger av ulike stimuli fra både eksterne faktorer som daglengde, og av interne faktorer som alder og energireserver. Det er også tidligere vist at etter at kjønnsmodnings-prosessen er påbegynt tidlig om vinteren, er tilstrekkelige energireserver i løpet av våren er en viktig faktor. Dette påvirker fortsettelsen av modningsprosessen, og utilstrekkelig nivå av energi eller fett om våren kan stanse videre utvikling. Hunnlaks er kjent for å investere mer energi i gonader enn hannlaks og hunnlaks blir normalt kjønnsmodne senere i livet enn hannlaks. - Oftest på høsten etter to eller flere vintre i sjøen. Kombinasjonen av disse faktorene påvirker i stor grad produksjonsbiologien til oppdrettslaks. Dette er fokus for denne avhandlingen.

I denne avhandlingen ble tetradecyltioacetetic acid (TTA) tilsatt strategisk i fôr til S0 laks for å endre akkumuleringen av muskelfett i løpet av den første våren i hav (artikkel I). Det er vist at lavere fett-akkumulering på våren, førte til at tidlig kjønnsmodning av hannlaks (grilse) etterfølgende høst ble redusert med en tredjedel sammenlignet med kontrollgruppen. Denne fikk samme fôr, men uten tilsetting av TTA. For å beskrive nedgangen av TTA i fiskemuskelen etter fôring med TTA, ble en eliminasjonsmodell utviklet i dette studiet (artikkel I). Siden hunnlaksene vanligvis kjønnsmodnes senere i livet enn hanner, ble et annet eksperiment med S1 laks utført for å undersøke mulig ulik respons mellom kjønnene mot TTA (artikkel II). Resultatene viste at fett i muskel hos hanner og hunner varierte vesentlig etter TTA-behandling. Innhold av muskelfett i løpet av den første våren var betydelig lavere hos hunner enn hos hanner. I løpet av den andre våren, var fettinnholdet betydelig mer redusert hos hanner enn hos hunner. Kondisjonsfaktoren fulgte et lignende mønster som utviklingen av muskelfett. Resultatene i artikkel II viser at forskjellen mellom kjønnene i fettakkumulerings-dynamikk sannsynlig er relatert til en kjønnsspesifikk forskjell i reproduksjonsbiologi hos atlantisk laks.

For å få bedre innsikt i respons på TTA-behandlinger, ble det valgt å studere laksens hjerte fordi hjertet er et svært energi-forbrukende og vitalt organ (artikkel III). Det er vist at fisk gitt fôr tilsatt TTA, hadde en mindre reduksjon i hjertevekt i forhold til fiskevekt (CSI). Dette ble funnet ved sammenlikning med kontrollgruppen i en periode etter utsett i sjøen. Dette

sammenfalt med redusert kondisjonsfaktor og redusert fett i muskler i den behandlede fisken. For å undersøke dette videre, ble hjerteceller isolert fra laks, holdt i kultur og pre-stimulert med TTA for endogent å øke konsentrasjonen av denne bioaktive komponenten. Radiomerket fettsyre ble tilsatt kulturmediet for å studere virkningen av endogent TTA på opptak, inkorporering i lipidklasser og β-oksidasjon. Det er vist at hjerte celler som ble stimulert med 120μM TTA hadde høyere opptak av radioaktivt merket fettsyre og dannelse av β-oksidasjonsproduktene CO2 og andre syreoppløselige produkter. De molekylære mekanismene som ligger til grunn for dette ble studert i et ytterligere eksperiment med laks holdt i kar på land. Disse ble innsprøytet med økende doser av TTA. Ved å studere genuttrykk (RT-PCR), er det vist at gener som regulerer cellevekst, peroksisomal fettsyre-oksydasjon, fettsyre-elongering og desaturering, ble oppregulert i hjertet av TTA-behandlet fisk. Derimot ble gener som er involvert i fettsyre-transport i mitokondrier ikke påvirket. Samlet sett viser funnene i artikkel III at TTA-behandling fører til økt hjertestørrelse, muligens på grunn av en økning i uttrykk av gener som regulerer hjertecelletilvekst og en forbedret energiproduksjon grunnet stimulering av fettsyre-oksydasjon.

Vi konkludere at funnene i denne avhandlingen viser at TTA i definerte doser, med fordel kan brukes strategisk under energikrevende perioder for atlantisk laks. Dette kan gjøres uten å påvirke fiskens vekst. I tillegg reduserer TTA forekomsten av tidlig kjønnsmodning, øker hjertets robusthet og β-oksidativ kapasitet i oppdrettslaks. Videre er vist at effekten av TTA kan påvirkes av kjønn og fiskestørrelse.

Implementering av kunnskapen fra arbeidet i denne avhandlingen har potensiale for å være nyttig for lakseoppdrettsindustrien.

List of publications

The thesis is based on the articles listed below and the articles will throughout the thesis be referred to by roman numerals in the text.

I. Arge, R., Thomassen, M.S., Berge, R.K., Zambonino-Infante, J.L., Terjesen, B.F., Oehme, M. and Rørvik, K-A. (2012). Reduction of early sexual maturation in male S0 Atlantic salmon (*Salmo salar* L.) by dietary supplementation of tetradecylthioacetic acid (TTA). Aquaculture Research 45, 1–12, DOI:10.1111/are.12036.

II. Dessen, J-E., Arge, R., Thomassen, M. S. and Rørvik, K-A. (2016). Differences in fat accumulation between immature male and female Atlantic salmon *Salmo salar* after dietary administration of tetradecylthioacetic acid. Journal of Fish Biology 89, 2085–2097. doi:10.1111/jfb.13113

III. Arge, R., Dessen, J-E., Østbye, T-K., Ruyter, B., Thomassen, M.S., Rørvik, K-A (2017). Effects of tetradecylthioacetic acid (TTA) treatment on lipid metabolism in salmon hearts – in vitro and in vivo studies. Journal of Fish Physiology and Biochemistry, https://doi.org/10.1007/s10695-018-0466-4

1. Introduction

1.1 Salmon aquaculture development

Since its beginning in Norway in the early 1970s, Atlantic salmon (*Salmo salar*, L.) aquaculture has expanded throughout the North Atlantic and North/South East Pacific as well as in Tasmania and has become a major industry in most of the involved countries (Jones 2017). The growth in production has implemented several factors whereof most important have been generally improved production, improved feed and feeding strategies, development of vaccines and genetic selection for better nutrient utilisation.

One of the improvements is hatchery control of temperature and photoperiod, which has abled the industry to produce both 'in season' smolt for seawater transfer in spring about 16 months after hatching (S1 smolt) and 'out of season' smolt for transfer in autumn (S0 smolt) about 8 months post hatching (Duncan *et al.* 2002). In recent years in Norway, the ratio of smolt transferred to sea in spring or autumn has been estimated to about 60/40 and this ratio may become more equal in future (Kittelsen *et al.* 2006) as the additional use of S0 smolts facilitates a more controlled year round production of marked-size salmon (Mørkøre & Rørvik 2001; Duncan *et al.* 2002).

1.2 Salmon energy metabolism

Utilisation of fatty acids highly dominates energy metabolism in high performance fish (Patton *et al.* 1975; Moyes *et al.* 1992; West *et al.* 1993; Castro *et al.* 2013) where energy demand in oxidative muscles such as red muscle and heart is mostly met by mitochondrial aerobic metabolism. In general, it appears that the mechanisms of fatty acid uptake, circulation and metabolism in fish are similar to mammals (Tocher 2003, Todorcevic *et al.* 2008, Torstensen *et al.* 2009). Thus, fatty acid transport to cells is facilitated by circulating lipoproteins where uptake may take place through passive diffusion over the cell membrane but especially by active uptake by membrane bound proteins. In cytosol, enzymes convert the fatty acids to acyl-CoA esters which enter the mitochondria via the carnitine palmitoyltransferase transport system and enter the β-oxidation pathway where each acyl-CoA is shortened by two carbons for each β-oxidation step. Peroxisomes are also involved in the catabolism of fatty acids, but do not contain an energy coupled electron transport. Peroxisomes contain flavin oxidases which catalyse the reduction of O_2 to H_2O_2 which again

is quickly reduced to H_2O by catalase. Especially very long chained acyl-CoAs are chain shortened and partially β-oxidised in the peroxisomes. The end products are transported from the peroxisomes to the mitochondria for further oxidation.

To promote rapid growth in farmed Atlantic salmon, commercial feeds normally contain high fat levels. Normal practise in farming of salmon in seawater, is thus to use relatively high inclusions of fat in the feed compared to protein from approximately 1 kg salmon body weight (≥ 35 % lipid), such that protein derived energy is spared in favour of fat. Hence, historically, the lipid inclusion has increased about four times since the start of the industry (Tacon & Metian 2009; Torrisen *et al.* 2011). In such a change, the feeds for salmon have gradually become denser in energy.

Being a poikilothermic species, feed intake and growth of salmon is highly influenced by water temperature (Brett 1979, Lega *et al.* 1992, Jobling 1997). In combination with day length, these factors are of importance regarding nutrient utilisation and retention in salmon (Mørkøre & Rørvik 2001; Lysfjord *et al.* 2004, Oehme *et al*. 2010, Alne *et al.* 2011). It has been shown that a few weeks following transfer to the sea in the spring, the fish seem to have increased energy demand. The fish may have poor condition factor and low levels of muscle fat in this period (Jobling *et al.* 2002, Lysfjord *et al.* 2004, Alne *et al.* 2011) and outbreaks of various viral or bacterial diseases often occur (Bowden *et al.* 2002; Rørvik *et al.* 2007 and Hjeltnes 2016). In contrast, the period from summer until late autumn is characterized by rapid growth and high fat deposition (Mørkøre & Rørvik 2001, Alne *et al.* 2011). However, the following first winter at sea may also be seen as an energy demanding period for Atlantic salmon, during which levels of muscle fat may fall due to an increased energy demand for maintenance at the low sea temperatures (Lega *et al.* 1992, Handeland *et al*. 2000, Mørkøre & Rørvik 2001). Thus, seasonal variations in temperature and day length affect growth and fat accumulation in salmon (Mørkøre & Rørvik 2001, Nordgarden *et al.* 2003, Oppedal *et al*. 2011). These factors, combined with disease outbreaks and internal energy status, significantly influence the production biology of farmed Atlantic salmon.

1.2.1 Salmon sexual maturation

Another significant factor influencing growth and fat deposition in Atlantic salmon, is the initiation of puberty and the development in sexual maturation. Atlantic salmon may become mature in the first autumn at sea (jack maturation, Duncan *et al.* 2002), second autumn at sea

(grilse maturation, Duston & Saunders 1999, Duncan *et al.* 2002) or in the autumn after two or more sea winters (Duston & Saunders 1999). In farming of salmon, sexual maturation before harvesting size, called early maturation, may vary between cohorts and may depend on effect of strain and age combined with size of the fish (Taranger *et al.* 2010). Initiation of puberty and sexual maturation is a complex process depending on different stimuli gained from both internal factors like age and state of energy reserves (Thorpe *et al.* 1990, Shearer & Swanson 2000, Alne *et al.* 2009a, Taranger *et al.* 2010) and external factors like photoperiod and abundance of feed (Thorpe *et al.* 1990, Taranger *et al.* 1998, 2010, Fjelldal *et al.* 2011).

It is shown that in nature, the maturation process in Atlantic salmon may be initiated during early winter in the preceding winter season (Oppedal *et al.* 1999, 2006) as to ensure an appropriate a seasonal timing of reproduction, providing for favourable conditions for the offspring (Taranger *et al.* 2010). The next step in the maturation process are appropriate energy or fat reserves during the following spring period, which is a major factor controlling this process. During this particular period, low energy or fat levels may arrest further progress (Thorpe *et al.* 1990, Rowe & Thorpe 1990, Kadri *et al.* 1996, Duston & Saunders 1999, Duncan *et al.* 2002). Especially female salmon must invest more energy in the development of gonads compared to males (Aksnes *et al.* 1986). Hence, females normally mature at a higher age than males, most commonly in the autumn after two or more winters in the sea (Duston & Saunders 1999). In this context, it is known that salmon spawning in a given autumn will show higher growth during the previous winter and spring than their non-maturing counterparts (Kadri *et al.* 1997) and then cease growing as spawning time commences (Aksnes *et al.* 1986).

1.3 Functional feed ingredients

Being one of the highest costs in fish farming, feed for Atlantic salmon has been continuously improved to meet the nutritional requirements of specific life cycle stage and health of the fish. This has involved optimising protein and lipid levels as well as application of new protein and lipid sources (Hixson 2014). Additionally, in the past decade or so, feed manufacturers have introduced a new concept of feeds named “functional” feed. In such feeds, different additives are exploited beyond their nutritional effect alone to improve baseline fish performance in general, but also specifically to support the health and stress resistance during challenging periods, ex sea transfer, pre-treatments or handling. A large variety of additives

are applied: Probiotics, prebiotics, immunostimulants, vitamins, nucleotides, minerals and plant/algal extracts (for a review see Tacchi *et al.* 2011). It has been shown that such nutrients act on the molecular level (Martin *et al.* 2003, Froystad *et al.* 2008, Leaver *et al.* 2008) opening for the opportunity to target specific responses in the fish (Müller & Kersten 2003) by influencing gene expression by activation or suppression of transcript factors (see review by Desvergne *et al.* 2006). Such approaches may have significant implications for future design of new feed formulations.

Figure 1. Molecular structure of tetradecylthioacetic acid (TTA). The molecular formula of TTA is $C_{16}H_{32}SO_2$.

1.4 Tetradecylthioacetic acid (TTA)

Originally, thia fatty acids were prepared for different purposes, as to study mechanisms of enzyme reactions, preparation of non-metabolisable fatty acid analogues and to obtain pharmacological effects. Chemical properties of thia fatty acids are similar to normal fatty acids, however their metabolism and metabolic effects differ (Skrede *et al.* 1997). TTA is a synthetic 16 carbon saturated thia fatty acid where the carbon atom in the β-position is substituted by a sulphur atom (Figure 1.) (Berge *et al.* 1989).

Uptake and transportation of TTA is generally believed to be similar to normal fatty acids caused by the close resemblance (Skrede *et al.* 1997). Incorporation of TTA is mainly found to take place in phospholipids and acylglycerols and tend to accumulate in tissues (Skrede *et al.* 1997). In contrast to normal fatty acids and due to the substituted sulphur atom (figure 2), normal catabolic pathway via β-oxidation is inhibited (Skrede *et al.* 1997). TTA can however be catabolised through ω/β-oxidation and then via sulphur oxidation, albeit at slow rates (Skrede *et al.* 1997). The biological effects of TTA is especially through its action as an agonist for peroxisome proliferator-activated receptors, the PPARs (Raspé *et al.* 1999, Bremer 2001, Westergaard *et al.* 2001, Grammes *et al.* 2012b). TTA has been shown to decrease plasma lipids, adipose lipid stores and to enhance transportation of fatty acids to the liver of mammals (Berge *et al.* 2002). The hypolipidemic and anti-adipogenic property of TTA has

been related to an increase of both number and size of peroxisomes and mitochondria leading to increased capacity for β-oxidation of fatty acids in mammalian hearts and livers (Berge *et al.* 1989, Bremer 2001, Wensaas *et al.* 2009).

1.4.1 Biological effects of TTA in salmonids

It has been shown that high inclusions of fat in diets for salmon may lead to undesired fat deposition around inner organs, arteriosclerosis and other life-style associated diseases similar to what is seen in mammals (Poppe & Taksdal 2000, Brocklebank & Raverty 2002). Hence, ways to induce optimal utilisation of dietary fat versus excessive storage have been sought by applying TTA. Moya-Falcon *et al.* (2004) found that TTA significantly changed fatty acid composition in phospholipids, triacylglycerols and free fatty acids in Atlantic salmon gills, heart and liver. Especially, the content of n-3 PUFA increased and the content of saturated fatty acids decreased in the phospholipid fraction of gills and heart. In contrast, in TTA treated rainbow trout (*Oncorhynchus mykiss* L.), Kennedy *et al.* (2007) did not find significant changes in lipid composition in liver or flesh. The changes in lipid composition after TTA treatment of salmon are suggested to be related to a higher oxidation of other fatty acids as TTA is reported to increase mitochondrial β-oxidation in salmon liver, heart and muscle (Moya-Falcon *et al.* 2004, Vegusdal *et al.* 2005, Kennedy *et al.* 2007, Alne *et al.* 2009ab, Grammes *et al.* 2012b). TTA has also been reported to have an immune-stimulatory effect in salmon macrophages (Grammes *et al.* 2011). In line with this, Rørvik *et al.* (2007) and Alne *at al.* (2009b) observed reduced mortalities in salmon during outbreaks of heart and skeletal muscle inflammation (HSMI) as well as infectious pancreas necrosis (IPN) in TTA treated salmon. The authors pointed at a combination of anti-inflammatory stimuli, better protein conservation and mobilisation and increase of available energy resources as possible reasons.

In experiments with fish, TTA doses above 0,05% dietary inclusion seem to indicate negative implications, as decreased growth and higher mortality with increasing doses has been observed (Moya-Falcon *et al.* 2004, Kleveland *et al.* 2006). The main reason seems to be accumulation of TTA degradation products, the sulphur oxygenated TTA-metabolites, in salmon kidney affecting morphology and reduced density of residential melanomacrophages (Moya-Falcon *et al.* 2004, Gjøen *et al.* 2007).

1.5 Cell cultures

Biological studies of animal tissues or cultures of cells in vitro is widely used in science. Among many are applications within stem cell and cancer research, production of vaccines and biopharmaceutical- and nutritional studies (Jedrzejczak-Silicka 2017). Cell for studies may be obtained from established cell lines or tissue isolates. The advantage of in vitro experiments is that the cells in question are kept in controlled, well defined environments without being exposed to systemic effects and possible interference from endogenous mechanisms that may counteract or conceal obtained effects in conventional animal experiments (Mitcheson *et al.* 1998). Another important benefit of cell culture studies is the reduction in the use of animals sacrificed for research purposes. Caution, is however, needed when extrapolating data from cultured cells to the whole animal, as small changes in cell integrity and culture conditions may influence results (Mitcheson *et al*. 1998).

In vitro studies are also useful tools in studies of salmon lipid metabolism (Nurmi & Vornanen 2002, Moya-Falcon *et al*. 2004, Todorcevic *et al.* 2008). In this thesis, lipid metabolism in isolated salmon primary cardiomyocytes was investigated as lipid is the major fuel for respiration in this highly energy-consuming and vital organ (Patton *et al.* 1975, Moyes *et al.* 1992, West *et al.* 1993, Castro *et al.* 2013). The usefulness of studying cell isolates as cardiomyocytes, is that they may provide a model for the principles of energy metabolism subjected to variable extracellular conditions (Taegtmeyer *et al.* 2016). Another consideration for studying heart cell lipid metabolism, was the knowledge that highest concentrations of TTA are found in hearts of mammals as well as in fish and it is therefore of interest to increase the knowledge how increasing doses of endogenous TTA influence cardiomyocyte energy metabolism (Skrede *et al.* 1997, Grammes *et al.* 2012a).

2. Objectives

The objectives of this thesis were to explore the biological effects of the fatty acid tetradecylthioacetetic acid (TTA) on farmed Atlantic salmon with focus on:

- Strategic supplementation of TTA in feeds for S0 salmon to reduce muscle fat level in spring and early male sexual maturation the following autumn – an in vivo study.

- Strategic supplementation of TTA in feeds for S1 salmon to reduce muscle fat during two different energy demanding periods – an in vivo study.

- Stimulation of fatty acid metabolism in salmon hearts by TTA - in vivo and in vitro studies.

3. Results and general discussion

3.1 Effect of TTA on muscle fat accumulation in Atlantic salmon

Strategic TTA supplementation in the feed for S0 salmon (477g at stocking) for about 8 weeks in the first spring at sea (*Paper-I*) significantly reduced accumulation of fat in the fish muscle. In May, the treated fish had about 0.7% less muscle fat compared to the control which had about 10.8%. This was an interesting finding as TTA treatment of salmon at this size had not been done before. Overall fish weight was about 700g at that time. In June and onwards muscle fat content became similar between the fish groups, implying that the biological effect of TTA decreased despite the fact that the TTA content in fish muscle was at its highest in June (see elimination model in 6.1.1). One explanation to this may be that fat retention in salmon is strongly regulated on the molecular level to increase from June to September (Alne *et al.* 2011), and perhaps this function counteracted a possible delay in the transcriptional response to the level of TTA in June as suggested by Alne *et al.* (2009b) and Grammes *et al.* (2012a).

In *Paper-II*, a similar experiment was conducted but with much smaller S1 salmon in the first spring at sea (105g at stocking). In addition, a second TTA treatment was done in February in the following winter at which time the fish had reached more than 2 kg bodyweight. As expected, based on a similar the study by Alne *et al.* (2009a), muscle fat content in the treated fish group was significantly lower than the controls the first spring at sea. It was surprising, however, to find that the reduction in the treated group was mainly caused by a significant reduction of muscle fat in treated females whereas treated males had a similar muscle fat percentage as the control fish of both sexes. In the second spring, after the second treatment, TTA treated fish of both sexes had significantly lower muscle fat compared to the controls. Interestingly, in this period treated males had significantly lower muscle fat vs treated females. Thus, TTA treatment, in this case through diet, interacted significantly with the sex of the fish. The validity of the observations on fat accumulation in *Paper II* were supported by corresponding changes in condition factor. The results may indicate that the observed different dynamics of fat accumulation between the sexes are probably related to the reproduction biology of Atlantic salmon (see 6.2).

In general, overall performance i.e. growth, feed conversion and mortality in treated fish was not different compared to not treated fish in the experiments described in *Paper-I* and *II*.

3.1.1 Elimination model of TTA in Atlantic salmon muscle

TTA was detected in fish muscle at the first sampling about two weeks after initiation of TTA feeding (42µg TTA/g) (*Paper I*). Approximately two weeks after the TTA feeding ended (first week of June) the mean value of TTA in fish muscle was 184µg TTA/g. After the TTA-feeding ended, the reduction of TTA in fish muscle was found to be closely related to the accumulation of day degrees. The feasibility of using this variable in the regression analyses in this study was connected to the performance of the fish. Hence, general metabolism, energy deposition and utilisation are factors that are temperature dependent in poikilothermic species like salmonids (Bureau *et al.* 2002). Degradation of a compound and increase of tissue mass (Robin *et al.* 2003, Jobling 2004) are two factors that can explain the reduction in TTA concentration in muscle seen in this experiment. For estimation of half-time ($t_{½\beta}$) of TTA in muscle an exponential model significantly explained 95% of the variability in TTA in fish muscle. Based on this model average half-time of TTA in the salmon muscle was estimated to about 700 day degrees.

A comparison between the half time model and the calculated decrease of TTA based on dilution only, shows that the difference between the two models was most pronounced in big fish in the last period of the experiment. At the end of the experiment the concentration of TTA in muscle levelled off in an asymptotic manner. Therefore, it would be difficult to predict the time when all TTA had been removed from the muscle tissue.

3.2 Effect of TTA on sexual maturation in Atlantic salmon

Altered photoperiod has in various studies been reported to strongly control sexual maturation in Atlantic salmon and this practice is also widely applied in the salmon farming industry. However, total control is seldom achieved (see review by Taranger *et al.* 2010) and other factors are in play, i.e. temperature, growth rate and especially energy stores during spring preceding full maturation the following autumn. In this context, it is known that salmon may show a so-called growth spurt in the spring preceding spawning in autumn (Taranger *et al.* 2010). Springtime has also in studies been described to be an energy demanding period for salmon (Mørkøre & Rørvik 2001, Alne *et al.* 2011). Thus, in *Paper I* and *II*, TTA was strategically added to the feed for salmon in the first spring at sea as well as in the winter preceding the second spring at sea. In *Paper I*, it was shown that lower early sexual maturation

in male S0 grilse in September was linked to reduced muscle fat in previous month of May obtained by a TTA treatment. Not only was incidence of fish with outer secondary characteristics related to lower maturation in treated fish, also a significant slower development in male gonads was detected. It is worth noting, that commercial practises in the use of additional light were applied in the experiment, but this did not totally prevent occurrence of early sexual maturation.

It is established knowledge that salmon males dominate in the proportion of fish entering puberty as parr, jacks or grilse (Taranger *et al.* 2010). However, in S0 fish no difference in muscle fat between the sexes was detected in May (*Paper I*) and negligible incidence of female maturation occurred later in the experiment. It is suggested that salmon females require a higher energy threshold in spring – related to a higher energy investment and energetic cost in reproduction in females compared to males (Aksnes *et al.* 1986, Jonsson *et al.* 1997, Hendry & Berg 1999). Taken together, the results in *Paper-I* support the hypothesis that reduced muscle fat stores in spring in S0 as seen in S1 salmon (Alne *et al.* 2009a), significantly influences occurrence of sexual maturation in autumn the same year.

In *Paper II*, S1 males seemingly did not respond to the TTA treatment during the first spring. This was unexpected, and any explanation is not readily evident. Very few fish, however, reached early maturation in the autumn (six in each group). The low response to TTA in this study compared to a previous study (Alne *et al.* 2009a) was perhaps due to a lower TTA inclusion level in the feed, as well as the fact that the sexual maturation processes in Atlantic salmon males may differ with age and size (Taranger *et al.* 2010).

As the experiment was expanded further to an additional TTA treatment of the S1 salmon the first winter in sea, the lipid level of fish of both sexes were significantly higher. In this context, at least some of the fish may be expected to reach some state of maturation the following autumn. The reason for the differences in response to TTA between sexes at the different time periods may have involved endogenous mechanisms. It has been shown in seatrout (*Salmo trutta* L.) that PPARα mRNA expression can be significantly higher in female in May than in the rest of the year (Batista-Pinto *et al.* 2009), whereas the level in males may not change. It was suggested by the authors that the PPARα in *S. trutta* females was under oestradiol modulation and further, that cross-talk between this and the oestrogen receptor may occur. If this also holds for Atlantic salmon, it is possible that this mechanism influenced the effect of TTA on females described in *Paper II*, but this needs more investigation.

The findings in the experiment with S0 salmon show that it is possible to influence early grilse maturation by a dietary approach. Reduced incidence of early grilse maturation which leads to downgrading at the slaughterhouses and lower harvest value, has important implications for the salmon farming industry as much is invested in fish of this size. In S1 salmon, the effect was greater when TTA was administered during the first spring, but the expansion of the experiment to the next spring, perhaps provided a better understanding on the relationship between fat accumulation and sexual maturation in salmon. In general, the results demonstrate the importance of considering both sex and season when studying fat dynamics and reproduction biology in Atlantic salmon.

3.3 TTA stimulation enhances utilisation of fatty acids in salmon hearts

In the experiment described in *Paper II*, development in heart weight relative to bodyweight, the cardio somatic index (CSI), was also investigated (described in *Paper III*). The experiment demonstrated that dietary treatment with TTA in a period after transfer to sea water enhances the ability of salmon postsmolts to maintain a significant higher CSI. In studies of rats, TTA has been shown to result in proliferation of liver mitochondria and peroxisomes and increased liver size (Berge *et al.* 1989). Similarly, in salmon given TTA supplemented diets, increased liver size has been documented (Kleveland *et al.* 2006). Induced proliferation of mitochondria and perhaps also peroxisomes is, consequently, most probably the explanation for the larger heart size after TTA feeding found in the study (*Paper III*).

Cardiac fatty acid metabolism in salmon has been sparsely investigated. Consequently, the possibility of studying short term effects of TTA on salmon heart by pre-treatments of cardiomyocytes in culture was of interest (*Paper III*). After three days of TTA stimulation, positive effects on fatty acid uptake and oxidation to both CO_2 and acid soluble products (ASP) were seen at the highest dose used. Numerically higher incorporation into cell lipids was further observed, but the relative amounts of fatty acids oxidized or stored as lipids did not change. Most of the stored radioactivity was recovered in the phospholipid fraction and less in triglycerides with increasing doses of TTA. This may be taken as an indication of cell proliferation and such a suggestion may further be supported by the gene expression results in the second in vivo small-scale experiment described in *Paper III*. The attempt to distinguish between effects on mitochondrial and/or peroxisomal beta-oxidation in cell cultures by analysing the production of different acid soluble products gave no clear answer. However,

injection of TTA (*in vivo II* experiment, *Paper III*) resulted in a clear stimulation of ACO transcription, while any effect on the mitochondrial CPT 1 transcription was not seen. This may suggest that at least the short time effect of TTA on fatty acid oxidation in salmon hearts mainly is due to an increase in peroxisomes and peroxisomal ß-oxidation capacity. On the other hand, the gene PGC1a was clearly upregulated in this study and indicated a stimulation of mitochondrial biogenesis and increased β-oxidation in this cell compartment (Jäger *et al.* 2007).

The peroxisome proliferator-activated receptors, the PPARs, have in studies with salmon been shown to be upregulated by TTA. Especially the expression of PPARα was shown to increase in salmon hearts after treated with TTA-feed for 8 weeks in sea (Grammes *et al.* 2012a) and a slight, non-significant increase in PPARβ was further observed. In our short-time study, the expression of neither PPARα nor PPARγ was enhanced by injection of TTA, PPARα was even negatively affected at the lowest dose. Conversely, TTA significantly increased the amount of PPARβ mRNA by all three applied doses. PPARβ is known to stimulate fatty acid oxidation in rat cardiomyocytes (Gilde *et al.* 2003). In addition, PPARβ has also been found to be related to physiological cardiac hypertrophy (Grammes *et al.* 2012a) which may explain the increase of CSI observed in the in vivo I experiment (*Paper III*). The activation of transcription factors like the NKX 2.5, PCNA and partly PGC1 seen in the injection study may also be taken to corroborate with this view.

Relative activity in polyunsaturated fatty acid synthesis seen as increased relative amount of mRNA derived from the elongation and desaturation genes Δ5, Δ6, Elovl2 and 5 as well as the sterol-binding proteins SREBP1 and 2, seemed higher in TTA-treated fish hearts. In rat hearts, a two-fold increase in 22:6 (n-3) and major decrease in 20:4 (n -6) have been found (Skrede *et al.* 1997). Similarly, Moya-Falcón *et al.* (2006) reported an accumulation of 22:6 (n-3) in cell membranes of salmon liver after TTA treatment. In the latter study, the authors related the accumulation to an increase in oxidation of other more utilisable fatty acids and thus a conservation of 22:6 (n-3) rather than an increase in desaturation and elongation of shorter chain n-3 fatty acids. But the results may, in addition to the higher capacity of energy utilisation, indicate that hearts in TTA treated fish are more robust and able to secure the need for healthy fatty acids.

Additionally, the investigated genes related to cell genesis/differentiation in this experiment were upregulated. The relative mRNA amount of 5' AMP activated protein kinase (AMPK)

seemed to be higher in TTA-treated fish, which may indicate lower energy status within the cell as compared to untreated fish. As noted above, lipid and protein synthesis seemed upregulated, thus, upregulation of AMPK may seem contradicting as the AMPK is believed to inhibit lipid synthesis when energy status within the cell is low (Castro *et al.* 2011; Polakof *et al.* 2011). On the other hand, AMPK may induce transcription or activate genes that are involved in protein synthesis (Hardie 2004) which perhaps can be interpreted as the role of AMPK in this experiment.

Taken together, the results described in *Paper IIII* seem to indicate a higher catabolic activity of fatty acids in the salmon heart as a response to TTA treatments. Such increase in cardiac efficiency may offer significant benefits for farmed Atlantic salmon, especially in energy demanding situations such as after transfer from freshwater to seawater as in the in vivo I experiment. This may also be related to the significantly higher survival previously observed in TTA treated S0 post-smolts during a natural outbreak of heart and skeletal muscle inflammation (Alne *et al.* 2009b).

Concluding remarks

The findings in this thesis demonstrate that TTA may be used as a strategic feed component for S0 salmon in a defined time interval during spring. Without compromising growth, the results showed that it is possible to reduce accumulation of fat in the fish muscle in a short period and that this reduction significantly reduces incidence of early sexual maturation in the succeeding autumn. The obtained reduction in incidence of unwanted early male maturation is of importance for the farming industry as maturation in salmon of about 3 kg is very costly.

After sea transfer, TTA enhanced the ability of salmon postsmolts to maintain higher CSI and thus an improved capacity of energy utilisation. The observation was strengthened by investigation of lipid metabolism in cultured salmon heart cells, where short time TTA stimulation had positive effects on fatty acid uptake and oxidation. Altogether, the results indicate that hearts of TTA treated fish are more capable which may be interesting in the context of salmon farming.

Future perspectives

The new knowledge gained by the work in this thesis, points at the importance of a dynamic approach in future feed formulation for farmed Atlantic salmon at sea, where fish size and season is considered. It is shown that a dietary approach resulting in an enhanced fatty acid uptake and oxidation and altered muscle fat accumulation during specific periods at sea, may have important application within the farming industry.

Although TTA to date is not approved as a feed additive, TTA was in this thesis shown to have profound positive effects and may thus serve as a model compound when investigating future feed additives for special purposes.

When exploring future feed additives aiming at mobilisation of stored fat or enhanced utilisation of dietary lipids, the magnitude of the influence by endogenous sex steroids needs to be evaluated. During the work of this thesis, it was observed that elevated level of at least β-estradiol may counteract previously described effects of TTA (unpublished). Controlled trials elucidating effects of sex steroids on fat accumulation in salmon may be informative and highly relevant.

Elevated robustness and performance of salmon hearts by a dietary approach as described in this thesis, is relevant for testing in future studies. Stimulation of cardiac performance prior to periods of handling of salmon in farming situations or energy demanding periods should be evaluated.

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Paper I

Paper I

Aquaculture Research

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Reduction of early sexual maturation in male S0 Atlantic salmon (*Salmo salar* L.) by dietary supplementation of tetradecylthioacetic acid (TTA)

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Abstract

Effects of dietary tetradecylthioacetic fatty acid (TTA) on muscle fat, development of gonads and early sexual maturation in S0 Atlantic salmon during the first year in sea were investigated. TTA (0.5% w/w) was added to the feeds for 8 weeks in the spring. In May, at the end of the TTA-feeding period, the fish in the TTA group had significantly ($P < 0.05$) less fat (10.1%) stored in muscle compared with the control group (10.8%). In September, mean male gonadosomatic index (GSI) in maturing fish in the TTA group was found to be lower compared with the maturing fish in the control group ($P = 0.05$). On the basis of GSI values, male sexual maturation in September was 10.0% vs. 14.4% for the TTA and the control group respectively. Thus, relative to the control group, the incidence of male sexual maturation in the TTA group was reduced by about 1/3 ($P = 0.002$). Production data was not affected by dietary supplementation of TTA. This study reveals that TTA significantly reduces the incidence of male sexual maturation in S0 Atlantic salmon. A significant elimination model of TTA in fish muscle that takes into account, the growth rate of the fish was further developed in this study.

Keywords: Atlantic salmon, sexual maturation, gonadosomatic index, TTA, fat, S0

Introduction

In the wild, Atlantic salmon (*Salmo salar* L.) may show variation regarding age and size at puberty both between and within strains, years and environmental conditions as reviewed by Taranger, Carrillo, Schulz, Fontaine, Zanuy, Felip, Weltzien, Dufour, Karlsen, Norberg, Andersson & Hansen 2010;. In commercial farming, Atlantic salmon has been shown to mature at an early stage 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 in the autumn after two or more sea winters (Duston & Saunders 1999). The process of initiation of sexual maturation of fish seems to depend on different stimuli gained from both internal factors like age and state of energy reserves (Thorpe, Talbot, Miles & Keay 1990; Shearer & Swanson 2000; Alne, Thomassen, Sigholt, Berge & Rørvik 2009a; Taranger *et al.* 2010) and external factors like photoperiod and abundance of feed (Thorpe *et al.* 1990; Taranger, Haux, Stefansson, Björnsson, Walther & Hansen 1998; Taranger *et al.* 2010; Fjelldal, Hansen & Huang 2011). It is known that salmon spawning in a given autumn will show higher growth during the previous winter and spring than their nonmaturing counterparts (Kadri, Thorpe & Metcalfe 1997) and then stop growing as spawning

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time commences (Aksnes, Gjerde & Roald 1986). In addition to reduced feed intake and weight gain (Aksnes *et al.* 1986; Kadri, Mitchell, Metcalfe, Huntingford & Thorpe 1996), sexual maturation in farmed salmon leads to economical losses by downgrading of the fish when slaughtered caused by changes in external characteristics and reduced muscle quality (Aksnes *et al.* 1986).

Normal practices in Norwegian smolt production have been to transfer 'in season' smolt to seawater in spring about 16 months after hatching (S1 smolt). However, during the past decade or so, 'out of season' smolt is produced (Duncan *et al.* 2002), and transferred to seawater in the autumn about 8 months post hatching (S0 smolt). In recent years, in Norway, the ratio of smolt transferred to sea in spring or autumn has been about 60/40 (Kittelsen, Rosten, Ulgenes, Selvik & Alne 2006). The use of S0 salmon also facilitates a more controlled year round production of marked-size salmon (Mørkøre & Rørvik 2001; Duncan *et al.* 2002).

In commercial farming, S0 salmon will normally spend one or two winters in sea depending on size demand from the market or strategy of the farmer. In farming of S1 salmon, photoperiod manipulation is frequently applied with the use of additional light to reduce grilse maturation (Endal, Taranger, Stefansson & Hansen 2000; Alne *et al.* 2009a). With S0 salmon, normal practice is to use additional light during the first sea autumn/winter/spring to increase the growth rate at this time of the year (Oppedal, Dempster & Stien 2011). Variable effects have been shown, however, on the use of different light regimes on reducing sexual maturation in the second autumn in sea (Duncan, Mitchell & Bromage 1999; Oppedal, Taranger, Juell & Hansen 1999; Taranger *et al.* 2010). Assuming even better husbandry and feed quality, one may anticipate that variable effects of light regimes and increased growth rate in future may cause an increase in proportion of early mature S0 salmon in autumn after one-sea winter. This may increase the economical losses for the farmers, as the S0 salmon second autumn in seawater have almost reached a marketable size compared with the much smaller size of S1 salmon first autumn in sea (McClure, Hammell, Moore, Dohoo & Burnley 2007).

The bioactive tetradecylthioacetic fatty acid (TTA) has become of interest as an additive in fish feeds in recent years (Moya-Falcón, Hvattum, Dyrøy, Skorve, Stefansson, Thomassen, Jakobsen, Berge & Ruyter 2004; Kennedy, Bickerdike, Berge, Porter & Tocher 2007; Rørvik, Alne, Gaarder, Ruyter, Måseide, Jakobsen, Berge, Sigholt & Thomassen 2007; Alne, Thomassen, Takle, Terjesen, Grammes, Oehme, Refstie, Sigholt, Berge & Rørvik 2009b; Alne *et al.* 2009a). In mammals, TTA has been shown to increase both number and size of peroxisomes and mitochondria leading to increased capacity for β-oxidation of fatty acids (Bremer 2001). TTA also decreases plasma lipids, adipose lipid stores and enhances transportation of fatty acids to the liver (Berge, Skorve, Tronstad, Berge, Gudbrandsen & Grav 2002). Supplementation of TTA in diets for farmed fish may have positive effects by increasing the capacity for β-oxidation of fatty acids (Moya-Falcón *et al.* 2004). Mobilization of stored energy in the fish and thereby an increase in available energy resources can be a beneficial feature during energy demanding periods for the fish (Rørvik *et al.* 2007; Alne, Oehme, Thomassen, Terjesen & Rørvik 2011).

In addition to day length (photoperiod), the level of the energy reserves in late winter or spring has been shown to be of importance, as to whether the fish will initiate a maturation process or not (Thorpe *et al.* 1990; Kadri *et al.* 1996; Duston & Saunders 1999; Alne *et al.* 2009a). Added to the feed a few weeks after sea transfer, TTA has been shown to reduce fat reserves in muscle in S1 salmon leading to a reduced incidence of jack maturation in the following fall (Alne *et al.* 2009a). A similar effect in S0 salmon could be particularly interesting due to the higher economical loss experienced with matured fish as compared with the costs when using the much smaller S1 salmon. The main objectives of this experiment were therefore to evaluate the effect of dietary TTA supplementation in a short period in the first spring on possible fat reduction, and development of gonads and early sexual maturation in S0 salmon second autumn in sea. As TTA is regarded as a bioactive component, it was also important to establish knowledge about the elimination of TTA in fish muscle, both with regards to the component as a feed additive and to the consumer's interests.

Material and methods

Fish material and experimental design

The fish were supplied by the salmon farming company Salmar, Norway. Atlantic salmon were transferred to sea in autumn 2007 as S0 smolt. In

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January 2008, the fish were transferred from the Salmar locality to the Nofima Marin research station at Averøy (63°N) on the west coast of Norway. On arrival, all fish were stocked in one cage, which was illuminated by two submerged 400 W light bulbs 24 h day^{-1}.

The experiment consisted of three periods: March to May 2008, May to September 2008 and September 2008 to February 2009. An overview of the experimental design is shown in Fig. 1. Throughout the experiment, sea temperature was measured at 3 m depth and recorded daily. Average sea temperatures were 7.2, 13.7 and 9.2°C in the three periods respectively.

Supplementation of TTA was an integrated part of a larger experiment and other dietary additives were also tested at the same time. In this study, issues concerning effects of dietary supplementation of TTA are presented.

Period 1: March–May 2008

On March 28th 2008, the S0 smolt was restocked into 18 cages with 350 fish per cage. The 18 cages were organized in two piers and three blocks (Pier B1- Block 1, Pier B2 – Block 2 and 3) using a randomized block design, where each block consisted of a replicate of each of six test diets. Average bodyweight per cage when restocked was 477 ± 1 g. The cages measured 5 × 5 × 5 m and were illuminated 24 h/day by one submerged 400 W light bulb per cage until end of May. Feeding was carried out using automatic feeders four times a day and waste feed was collected as described in Einen, Mørkøre, Rørå and Thomassen (1999) and analysed for recovery of dry matter as described by Helland, Grisdale-Helland and Nerland (1996). The fish were fed to satiation and daily feed ration adjusted accordingly. In this period, the fish were given six different diets in triplicates, where three diets were supplemented with TTA (TTA 1–3) and three were not supplemented with TTA (Control A–C; see section Diets.). Supplementation of TTA in the feed stopped on May 24th or 25th 2008 when the fish in the different cages had eaten 2 ‰ TTA of initial biomass. Pellet size in this period was 7 mm.

Period 2: May–September 2008

Fish groups initially given TTA supplemented diets in period 1 were from end of May to September 10th 2008 given diets without TTA. The other fish groups were kept on the same diets as in period 1. Feeding was carried out as described in period 1. Due to increasing bodyweight of the fish, the pellet size was changed from 7 to 10 mm in first week of July.

Period 3: September 2008-February 2009

In September 2008, the original project ended. However, for registration of any delayed sexual

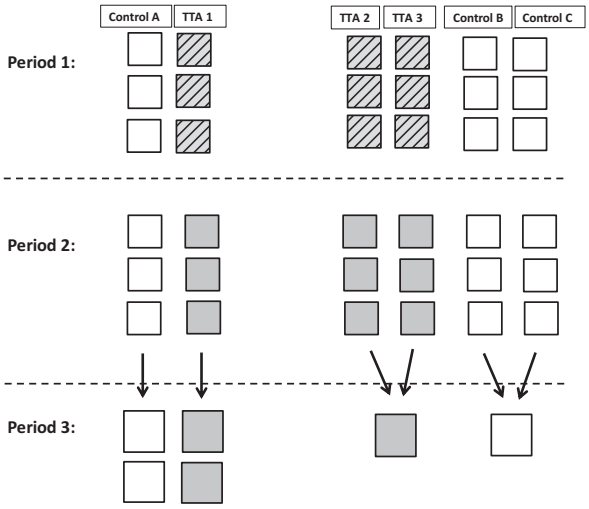

Figure 1 Schematic overview of the TTA-experimental design. Period 1 (March–May 2008): Grey filled squares with diagonal lines represent cages where S0 salmon were given TTA supplemented diets. Period 2 (May–September 2008): Stop of TTA feeding. Grey filled squares without diagonal lines represent cages where the fish were given TTA supplemented diets in period 1. Period 3 (September 2008–February 2009): Transfer of fish from 5 × 5×5 to 7 × 7×7 m cages, all fish groups were given the same commercial diet. (See sections Fish material and experimental design).

maturation, final washout of TTA in the muscle and production data of market size salmon (5 k), all fish were restocked and the experiment was extended for an additional 4 months period.

Fish in the triplicates that initially in period 1 had been given the diets Control A or TTA 1 were restocked into duplicated $7 \times 7 \times 7$ m cages. After restocking, the number of fish in these cages were 185/186 and 191/192 for the Control and TTA respectively. Feeding was carried out using automatic feeders, and waste feed was collected as described above and daily feed ration adjusted accordingly.

Two other $7 \times 7 \times 7$ cages were used for restocking of the rest of the fish, i.e. one single cage was used for all fish originally given, the TTA supplemented diets TTA 2 or TTA 3 ($n = 862$) and the other for fish given the nonsupplemented TTA diets Control B or Control C ($n = 733$). The fish in these two cages were fed by appetite with no waste feed collection.

In this period, all fish were fed the same commercial diet, based on the same formulation as in the 10 mm pellet of the diet Control A.

Sampling and fish analyses

Period 1 and 2, 2008

A total of 9 samplings were performed in period 1 and 2 (Table 1). In each sampling, 10 (nonmaturing) fish per cage were collected, representing the overall mean weight of the respective cage. During the experiment, bulk-weighing of the fish was not carried out on all samplings, but in time intervals as shown in Table 1. The sampled fish were anaesthetized (MS-222 metacaine 0.1 g L^{-1}, Alpharma, Animal Health, Hampshire, UK.) and then killed by a blow on the head. Length, bodyweight and sex of each sampled fish were recorded. Sex was determined by visual inspection of gonads. The fillet from the NQC-cutlet (NS 9401 1994) was photographed and fat percentage in cutlet was predicted by digital image analyses carried out using PhotoFish^{TM} (Folkestad, Wold, Rørvik, Tschudi, Haugholt, Kolstad & Mørkøre 2008). Thereafter, the fillets were frozen at −20°C for later analyses of TTA content. As this experiment was a part of a larger project, the sampling dates were fixed beforehand. Therefore, the first sampling for TTA measurements in the fish muscle was not performed exactly on the day for termination of TTA feeding, but about 10 days later. Analyses of fillet (NQC-area) for accumulation and elimination of TTA in fish fed the diets Control A or TTA 1 were carried out as described in Kennedy *et al.* (2007) at the Institute of Medicine, Haukeland University Hospital in Bergen, Norway.

In the August sampling, fish classified as maturing based on external characteristics were removed from the cages and visually checked for gonad size. In September, a more thorough registration of gonads was carried out: All visually classified maturing salmon as well as the 10 fish cage^{-1} (nonmaturing) sampled for analyses, were killed, weighted and gonads taken out and weighted.

Period 3: September 2008 – February 2009

The fish were recorded for sexual maturation and sampled twice during September 2008–February 2009 (Table 1). Fat and TTA in the muscle of 10 fish from each of the duplicated cages were analysed as described in period 1 and 2. In addition, fish visually classified as maturing based on secondary characteristics were visually checked for gonad size and removed from the cages.

Diets

All experimental diets were based on commercial diets (CPK500 or CPK 1000) produced by Biomar, Brande, Denmark. Supplementations of additives were all carried out by the feed producer. As part of a greater dietary study the bioactive tetradecylthioacetic fatty acid (TTA) was added in combination with different amino acids (Table 2).

TTA was added to three out of the six diets (TTA 1–3). Diet TTA 1 was added 0.5% (w/w) TTA, whereas to diet TTA 2, a combination of the amino acids Arginine (Arg, 1.1% w/w) and Glutamate (Glu, 0.75% w/w) was also added. In diet TTA 3, the amino acid Histidine (His, 1.0% w/w) was also added. Thus, the TTA diets, all had the same level of TTA, but different supplementation of amino acids (TTA 1 = 0.5% TTA, TTA 2 = TTA 1 + Arg/Glu and TTA 3 = TTA 2 + His). Dietary TTA was administrated from the start of the experiment until 2‰ (w/w) TTA of initial biomass in each cage had been eaten (Period 1). In period 2, TTA was substituted by carnitine (0.20% w/w).

Control A was the only nonsupplemented diet, whereas Control B was added the same dietary levels of the amino acids Arginine and Glutamate as in TTA 2 and TTA 3. Finally, one diet (Control

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Table 1 Overview of sampling and bulk-weighing dates during the experiment

Sampling dates						
Period 1	28.03.08	23.04.08	14.05.08			
Period 2	04.06.08	18.06.08	09.07.08	30.07.08	20.08.08	10.09.08
Period 3	11.11.08	02.02.09				
Bulk-weighing dates						
Period 1	14.05.08					
Period 2	18.06.08	11.08.08	10.09.08			
Period 3	02.02.09					

Table 2 Proximate composition of the experimental diets (7 and 10 mm) and the additives used in period 1 and 2 (no additives used in period 3, see text)

Pellet size 7 mm (CPK500)	**Control A**	**TTA 1**	**TTA 2**	**TTA 3**	**Control B**	**Control C**
Proximate composition (w/w):						
Crude protein (N×6.25)%	39.5	39.1	41.5	41.8	41.9	42.0
Lipid%	28.1	28.8	28.3	27.9	28.3	27.3
Ash%	7.7	8.1	8.2	7.9	7.9	7.8
Starch%	6.1	6.0	6.2	6.1	6.1	5.9
Dry matter%	92.6	93.0	94.1	93.1	93.9	93.5
Energy (MJ kg^{-1})	23.5	23.6	23.6	23.2	23.5	23.3
	36.4	40.4	42.1	39.8	40.8	41.7
Astaxanthin (mg kg^{-1})						
Pellet size 10 mm (CPK1000)	Control A	TTA 1	TTA 2	TTA 3	Control B	Control C
Proximate composition (w/w):						
Crude protein (N×6.25)%	37.3	37.1	38.5	38.8	38.8	40.7
Lipid%	32.7	32.7	31.5	31.4	31.4	30.8
Ash%	7.2	7.4	7.1	7.5	7.4	7.2
Starch%	6.7	7.0	7.3	7.7	7.1	7.1
Dry matter%	93.6	94.0	94.3	94.3	93.9	94.5
Energy (MJ kg^{-1})	24.2	24.3	24.2	24.1	24.3	23.9
	44.7	45.2	43.9	52.4	53.2	41.4
Astaxanthin (mg kg^{-1})						
Additives in period 1 (% w/w)*:	Control A	TTA 1	TTA 2	TTA 3	Control B	Control C
TTA	0	0.50	0.50	0.50	0	0
Glutamate	0	0	0.75	0.75	0.75	0
Arginine	0	0	1.10	1.10	1.10	0
Histidine	0	0	0	1.00	0	0
Glycine	0	0	0	0	0	0.43
Carnitine	0	0	0	0	0	0
Additives in period 2 (% w/w)*:	Control A	TTA 1	TTA 2	TTA 3	Control B	Control C
TTA	0	0	0	0	0	0
Glutamate	0	0	0.75	0.75	0.75	0
Arginine	0	0	1.10	1.10	1.10	0
Histidine	0	0	0	1.00	0	0
Glycine	0	0	0	0	0	0.43
Carnitine	0	0.20	0.20	0.20	0	0

*Expected levels according to composition of diets, not analysed.

C) was made iso-nitrogenous to Control B by addition of the amino acid Glycine (0.43% w/w).

All together, the different diets were designed to study potential additive, combined or synergetic effects of TTA and the different amino acids. In the case of no significant dietary effects of the supplemented amino acids, when evaluating TTA, pooled data from the TTA group (TTA 1–3) could be tested against pooled data from the control group (Control A–C).

When the original experiment ended in September 2008, all fish were given a commercial Biomar

diet (CPK1000) until the final termination of the trial in February 2009 (Period 3).

The feeds were analysed for dry matter (105°C until constant weight), ash (550°C until constant weight), nitrogen (Kjeltec Auto Systems, Tecator, Sweden) and energy (Parr 1271 Bomb calorimeter, Parr, USA). Crude fat was analysed as described by Folch, Lees and Sloane-Stanley (1957). Starch in the feeds was analysed as glucose after enzymatic hydrolysis employing a commercial kit (K-TSTA 05/06, Megazyme, Australia). Astaxanthin was analysed as described in Ytrestøyl, Struksnæs, Rørvik, Koppe and Bjerkeng (2006).

Calculations

The gonadosomatic index (GSI) was calculated as GW (g) · BW (g)$^{-1}$ · 100, where GW is the weight of the gonad in gram and BW is the total bodyweight of the fish in gram.

Growth measured as the thermal growth coefficient (TGC) was calculated as

1000 · ($BW_{d1}{}^{1/3}$ – $BW_{d0}{}^{1/3}$) · (Σ T)$^{-1}$ (Cho 1992), where BW_{d1} is the final bodyweight, BW_{d0} is the initial bodyweight and Σ T is the sum of day degrees in the period. Feed conversion ratio (FCR) was calculated as (kg feed eaten) · (kg final biomass−kg initial biomass + kg dead fish)$^{-1}$.

Statistical analyses

Statistical analyses of production data, fat, GSI and maturation were performed using SAS software (SAS Institute 1990). Initially, all data were analysed using analyses of variance (ANOVA) where the model used block, pier and dietary treatment as class variables. Experimental units were cages. As the statistical evaluation of the dietary treatments showed that the increased levels of amino acids in the diets, block or pier did not have any significant effect on the traits studied i.e. muscle fat, GSI and sexual maturation, the dietary treatments were pooled into one TTA and one control group, resulting in nine replicates per treatment. Frequency distributions of GSI levels between the two groups were analysed using Chi-squared tests.

Accumulation and elimination phases of TTA in the muscle were analysed by multiple regression analyses using STATGRAPHICS CENTURION XVI software (16.0.07 version). Elimination half-time ($t_{½\beta}$) of TTA in fish muscle was calculated using the equation ln(2)/β where β is the slope of the exponential function (Bryan, Zimmerman & Berry 1990).

The proportion of the total variation explained by models is expressed by R^2 and calculated as the marginal contribution of the mean square of the parameter (type III sum of squares). Significance level was set to $P \leq 0.05$ for all analyses. Results are presented as means ± SEM (standard error of the mean) if not specifically stated otherwise.

Results

No significant effect of the six dietary treatments *per se* was observed (see Statistical analyses). Similarly, no significant differences were observed in this experiment among the three diets supplemented in period 1with TTA (TTA 1–3), or among the three diets not supplemented with TTA (Control A–C). This means that in our study, increased dietary levels of the amino acids arginine and glutamate, and histidine or glycine, irrespective of supplementation of dietary TTA, had no effect on the traits studied. Therefore, the best possible evaluation of any effects of dietary supplementation of TTA first spring in sea in S0 salmon was to pool replicates given TTA, and replicates not given TTA (Control).

Production data

No significant difference was found in bodyweight between the dietary treatments throughout the experiment. At stocking in March 2008, the overall average bodyweight of the fish was 477 ± 1 g, at the end of period 1 in May: 702 ± 6 g, at the end of period 2 in September: 2580 ± 14 g and finally at termination of the experiment in February 2009: 5073 ± 21 g (only Control A or TTA 1). Overall average mortality throughout the experiment was 2.3 ± 0.4%.

No significant difference was registered in thermal growth coefficient (TGC) between the fish groups from March to September or between the two dietary groups (Control A and TTA 1) placed in the 7 × 7 × 7 m cages in period 3. Overall average TGC from March to September 2008 was 3.38 ± 0.01 and 2.73 ± 0.06 from September to February 2009. Weighted average TGC for the whole experiment was 3.01 ± 0.03.

Similarly, no significant difference was observed regarding feed conversion ratio (FCR) between the

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fish groups in the experiment. Average FCR from March to September 2008 was 0.97 ± 0.00 and from September 2008 to February 2009 1.05 ± 0.02. Overall weighted FCR was 1.01 ± 0.06.

Muscle fat

Fat in muscle (NQC) increased from approximately 8% at stocking in March to 16% at the end of the experiment (Fig. 2). No significant difference in muscle fat was found among the fish group given TTA supplemented diets (TTA 1–3) or among fish group given nonsupplemented diets (Control A–C) at any sampling time. Likewise, no significant effect of increased dietary levels of arginine and glutamate (TTA 2, TTA 3 and Control C) vs. diets not supplemented with these amino acids was observed. To evaluate any effect of TTA, the fish were therefore statistically pooled in two groups either given TTA supplemented diets (TTA group) or diets not supplemented with TTA (control group). By testing all sampling points, the only significant difference in muscle fat was observed in the May sampling ($P < 0.05$, R^2=0.28) (end of TTA feeding in period 1), where fish given TTA supplemented diets had significantly lower level of muscle fat (10.1 ± 0.1%) compared with the fish in the control group (10.8 ± 0.1%).

GSI and sexual maturation

In August and September 2008, early sexually maturing salmon were initially picked out based on secondary visible characteristics. In total, 460 fish were selected to be sexually maturing, 34 in August and 426 in September, where only two were identified as maturing females.

Gonadosomatic index (GSI)

The 95% confidence interval for GSI in fish sampled as immature males in September 2008 was 0.03–0.12% (n = 67 immature males, all diets). The GSI for the fish sampled as maturing males (all diets) was found to be between 0.07% and 12.36%. Fish selected as maturing based on visual inspection, but having GSI's within the GSI interval of immature fish, were excluded from the data for the sexual maturing fish group (n = 8). Significant lower mean GSI's were found in the pooled fish group given TTA supplemented diets (5.3 ± 0.2%, n = 170) than in the control group (6.1 ± 0.2%, n = 246) ($P = 0.05$, $R^2 = 0.21$).

The distribution of GSI's in the TTA group was found to be numerically different from the Control when grouped in five intervals (χ^2=8.85, df = 4, $P = 0.06$) (Fig. 3). By reducing the intervals to four by grouping the two lowest frequencies (8–10% and > 10%), the distribution became significantly different (χ^2=8.12, df = 3, $P = 0.04$). A further test within the five GSI intervals (Fig. 3) revealed that the two end intervals < 4% and > 10% were significantly different (χ^2=6.08, df = 1, $P = 0.01$). Thus, S0 salmon fed TTA supplemented diets had a significantly higher share of smaller gonads and a lower share of larger gonads than the control group.

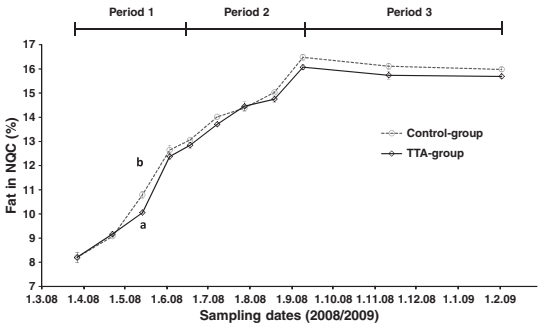

Figure 2 Muscle fat (%, w/w) (NQC) in S0 Atlantic salmon throughout the experimental period from March 2008 to February 2009. The figure presents fish groups either given diets with (TTA group) or without diets supplemented with TTA (Control group). Error bars are the standard errors of the mean. Significant difference between the TTA and the Control group in May is indicated by different letters.

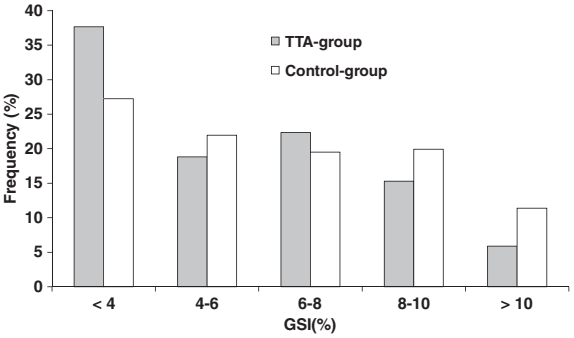

Figure 3 Frequency distribution of gonadosomatic index (GSI) of maturing Atlantic S0 salmon sampled in September 2008 (end of period 2). The fish groups were either given diets supplemented with TTA or control diets without TTA in period 1.

Sexual maturation

Visual inspection of S0 salmon in August revealed significant different maturation incidence between the TTA and the control groups ($P = 0.05$, $R^2 = 0.21$). Incidence of sexual maturation in August was 0.54 ± 0.31% ($n = 10$) and 1.47 ± 0.31% ($n = 24$) for the TTA and control group respectively. On the basis of GSI values in September, a significantly lower incidence of maturing males (Fig. 4) was found in fish group given TTA supplemented diets than in the control group ($P = 0.002$, R^2=0.47). Incidences of sexual maturation in the September sampling were 10.0 ± 0.5% ($n = 170$) and 14.4 ± 1.0% ($n = 246$) for the TTA and the control group respectively.

To check whether the observed significant difference in maturation was a real reduction or only a delay in the TTA group, the fish were kept separately and visually inspected in November 2008 and February 2009 (period 3). Six mature males of the total of 774 S0 salmon were found in the 7 × 7 × 7 m cages that held fish initially given diet TTA 1 or Control A – one in the TTA group and five in the control group. The two other cages, which held fish initially given either TTA 1 and TTA 2 or Control B and Control C (Fig. 1) was also visually inspected in February 2009. The incidence of maturation in these cages in February was 3.4% ($n = 29$) and 7.1% ($n = 51$) for the TTA and the control group respectively.

TTA content in muscle

TTA was detected in fish muscle at the first sampling about 2 weeks after initiation of TTA feeding (42 ± 7 μg TTA g^{-1}) and after about 5 weeks (May 14th 2008), the TTA level in the muscle had increased to 129 ± 17 μg TTA g^{-1}. Approximately, 2 weeks after the TTA feeding ended (first week of June), the mean value of TTA in fish muscle was found to be 184 ± 6 μg TTA g^{-1}. TTA was not detected in any sampling from the control group. No sulphur oxygenated TTA metabolites (TTA-SO or TTA-SO_2) were detected in the muscle of any of the fish groups.

In September, TTA in the muscle had decreased to 58 ± 6 μg TTA g^{-1} and further to 20 ± 4 μg TTA g^{-1} at termination of the experiment in February 2009. In the regression analyses of elimination of TTA in fish muscle, we used the variable day degrees (DG), which we set to accumulate from the start of the decline period. For estimation of half-time of TTA in muscle, the exponential model TTA= Exp (5.187–0.001·DG) explained 95% of the variability in TTA in fish muscle ($P < 0.001$) after transforming data to a reciprocal scale for linearization of the model (Fig. 5). On the basis of this model, average half-time ($t_{½\beta}$) of TTA in the salmon muscle was estimated to 696 ± 51 day degrees.

A comparison between the half-time model and the calculated decrease of TTA based on dilution only, shows that the difference between the two models was most pronounced in big fish in the last period of the experiment (Fig. 5).

Discussion

Overall performance of the fish in this experiment was good and production parameters, such as

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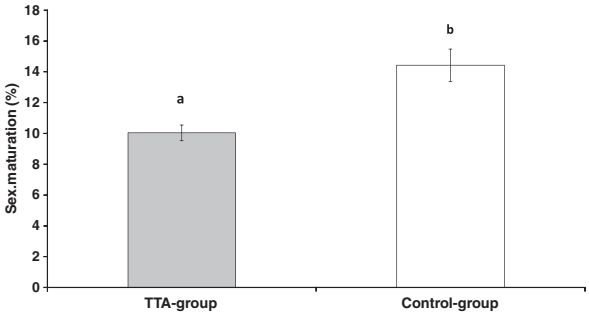

Figure 4 Percentage of sexual maturing S0 Atlantic salmon males in September 2008 based on GSI values. The fish were initially given diets supplemented with TTA or control diets without TTA in period 1. Error bars are the standard errors. Significant difference is indicated by different letters.

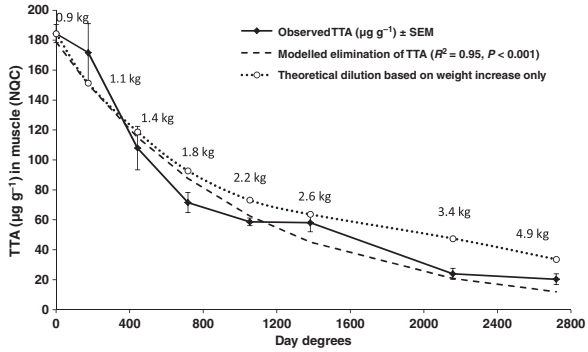

Figure 5 Observed and modelled elimination of TTA (µg g^{-1}) in the muscle of S0 Atlantic salmon in the experiment. Dotted line with circles represents a theoretical reduction based on weight increase alone. Fish bodyweight is shown above the line.

growth, feed conversion and mortality, were not found to be negatively affected by dietary supplementation of TTA.

When analysing the data, no significant effects of increased levels of the amino acids were observed. This means that in this study, the increased dietary levels of the amino acids arginine, glutamate and histidine, irrespective of supplementation of dietary TTA, had no effect on the traits studied. In addition, as no significant differences were observed among the three diets supplemented with TTA (TTA 1–3) or among the three diets not supplemented with TTA (Control A–C), the best possible statistical evaluation of TTA was to pool replicates in the TTA group and replicates in the control group, giving nine replicates per treatment. Using this analytical procedure, we found a significant reduction of 1/3 in sexual maturing frequency in the TTA fish group compared with the control group in September. This shows a positive effect of dietary TTA supplementation on incidence of sexual maturation in S0 salmon males, which will be of economical importance for the salmon industry. Furthermore, due to continuously improved breeding, nutrition and farming conditions, we believe that early sexual maturation may be an even greater problem in world wide salmon farming in the years to come, especially for S0 salmon in the autumn after about 1 year in sea. At that time, the S0 salmon may not have reached normal market size, but still the weight of about 3–4 kg makes early sexual maturation of S0 salmon costly for the farming industry, due to strongly reduced growth, increased mortality and downgrading/discarding of the fish due to low quality. For a scientific evaluation of the validity of the data with regard to biological consequences for the farming industry, we considered it important to follow the S0 salmon for about 4 extra months for evaluation of the results, verifying that salmon fed TTA diet did not sexually mature more frequently later in the autumn compared with the control group.

The occurrence of sexual maturation in S0 salmon in this study (10–14%) was similar to the observed maturation in Duncan *et al.* (1999), but

higher compared with the reported 1% in Duncan, Auchinachie, Robertson, Murray and Bromage (1998) and < 4% in Oppedal, Berg, Olsen, Taranger and Hansen (2006). Considering that the maturation rate of males in this study can be regarded as high, it can partly be ascribed to the small sea cages used and relatively high observed specific growth rates of the fish (Endal *et al.* 2000). However, it has to be pointed out that a similar incidence in maturation was reported in the commercial farming of salmon of the same origin comparable with the Control used in this experiment (Rørvik pers. comm.).

In general, the GSI interval for maturing S0 males in the present study was comparable with the reported interval in the study of Duncan *et al.* (1999). Mean GSI in the TTA group was significantly lowered compared with in the control group. In addition, S0 in the TTA group had significantly greater proportion of individuals with relatively small gonads compared with the control group. Taken together, this may indicate that in addition to reduced unwanted sexual maturation, supplementation of TTA in the diet probably also induces a delay in development of gonads in sexually maturing S0 salmon. It may be speculated if this delay also postponed deterioration of flesh quality (Aksnes *et al.* 1986). However, flesh quality of maturing fish was not investigated in this experiment.

Although we applied commercial practices in the use of additional light in the cages first autumn/winter in sea, we did not find that this prevented occurrence of sexual maturation in the S0 salmon second autumn in sea in this study. Additional light may improve growth of small 0 + salmon in mid-winter without increasing the proportion of maturing fish next autumn (Oppedal *et al.* 1999; Taranger *et al.* 2010); however, variable effects have also been seen (Duncan *et al.* 1999; Taranger *et al.* 2010). This supports the hypothesis that other mechanisms in addition to day length contribute to the onset of sexual maturation (Thorpe *et al.* 1990; Taranger *et al.* 2010).

Our results agree with findings that the level of stored energy in the fish is a contributing factor for the onset of maturation in Atlantic salmon (Thorpe *et al.* 1990; Kadri *et al.* 1996; Duston & Saunders 1999; Alne *et al.* 2009a). In the study of Alne *et al.* (2009a), small S1 salmon given a similar dose of dietary TTA showed a relative reduction in muscle fat (NQC) of about 12% and a relative reduction of about 30% in incidence of jack maturation compared with a Control. Similarly, in small Chinook salmon (*Oncorhynchus tshawytscha*), Shearer and Swanson (2000) reported that level of whole body lipid in spring influenced maturation frequency in males next autumn. Considering the salmon used in the mentioned studies were small, our results show similar effect on maturation in bigger S0 salmon males obtained with a lower relative reduction (7.4%) of muscle fat in spring.

The results in this study may reveal more information about a level of a suggested energy reserve threshold (Rowe, Thorpe & Shanks 1991; Shearer & Swanson 2000) in muscle of S0 salmon first spring in sea – above which a higher incidence of sexual maturation may be expected. Established knowledge shows that a frequency dimorphism in maturation exists between males and females, as males typically dominate in the proportion of salmon entering puberty as parr, jacks or grilse (Taranger *et al.* 2010). Even though no difference in fat percentage was found between the sexes in this experiment, maturing females only represented about 0.5% of the selected maturing fish. Therefore, it may be possible that the energy threshold in spring is higher for S0 females than for males. This may be linked to a higher energy investment and energetic cost in reproduction in females compared with males (Aksnes *et al.* 1986; Jonsson, Jonsson & Hansen 1997; Hendry & Berg 1999). However, salmon can show great variability in size and age at puberty between years (Taranger *et al.* 2010).

After the TTA-feeding ended, the reduction of TTA in fish muscle was found to be closely related to the accumulation of day degrees. The feasibility of using this variable in the regression analyses in this study is connected to the performance of the fish. General metabolism, energy deposition and utilization are factors that are temperature dependent in poikilothermic species like salmonids (Bureau, Kaushik & Cho 2002). As this experiment was part of a larger project, the sampling dates were fixed beforehand. Therefore, we do not have data on TTA in muscle at the exact time when TTA feeding ceased. Degradation of a compound and increase of tissue mass (Robin, Regost, Arzel & Kaushik 2003; Jobling 2004) are two factors that can explain the reduction in TTA concentration in muscle seen in this experiment. One of the assumptions in a dilution model is that there is no degradation of TTA in the period. Due to the higher specific growth rate for small than for bigger fish in this study, dilution is more pronounced in period 2 than in period 3. At the end of

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the experiment, the concentration of TTA in muscle approached 20 μg g^{-1} in an asymptotic manner. Therefore, it would be difficult to predict the time when all TTA had been removed from the muscle tissue. However, assuming European costumers daily intake of 50 g of salmon (FAO Fisheries and Aquaculture Information and Statistics Service 2008), the intake of TTA would equal 1mg. In the study of Pettersen, Salem, Skorve, Ulvik, Berge and Nordrehaug (2008) 18 subjects/trial persons were given daily doses of 200–1000 mg TTA for seven consecutive days. The authors concluded that TTA at its highest dose was well tolerated by humans; however, studies of long time effects are needed.

Taken together, our results strengthen the hypothesis that in S0 as for S1 reduced salmon muscle fat stores in spring may significantly influence occurrence of sexual maturation in autumn the same year. In this experiment, we managed using a dietary approach to reduce the accumulation of muscle fat in S0 salmon first spring in sea without any overall negative effect on growth or mortality. Consequently, we found a significantly lower incidence of sexual maturation in S0 males the following autumn. In addition, the relatively lower degree of development in male gonads in the TTA group may also indicate a possible prolonged grow-out phase for this fish group.

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Paper II

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Differences in fat accumulation between immature male and female Atlantic salmon *Salmo salar* after dietary administration of tetradecylthioacetic acid

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This study provoked sex-specific differences in fat metabolism in Atlantic salmon *Salmo salar*, by dietary administration of tetradecylthioacetic acid (TTA) during their first spring and winter in the sea. The effects of TTA were evaluated in June of the first spring and May of the second spring in the sea, by analysing white muscle-fat content. Muscle fat in males and females differed significantly as a result of TTA in their diet and diet interacted with the sex of the fish. The fat content during the first spring after dietary TTA was lowered by a greater amount in females than in males, 3·1–4·3%, respectively ($P < 0·05$). In contrast, during the second spring, fat content was lowered by a greater amount in males than in females, 15·8–16·7%, respectively ($P < 0·01$). Condition factor followed a similar pattern to the muscle fat. The results indicate that the difference in male and female fat accumulation dynamics is related to sex-specific reproduction biology of *S. salar*. In addition, the findings show that it is important to consider the sex of the fish and the season of the year when studying fat dynamics and reproductive biology of *S. salar*.

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Key words: condition factor; reproduction biology; S1; smolt.

INTRODUCTION

Fishes must accumulate sufficient energy for sexual maturation and reproduction (Saborido-Rey & Kjesbu, 2005; Marshall & Browman, 2007). In salmonids, the transition between fresh water and sea water influences the dynamics of growth, the accumulation of fat and the initiation of the maturation process (Taranger *et al.*, 1999; Oppedal *et al.*, 2011). Consequently, the size and age at puberty varies widely in salmonids, as reviewed by Taranger *et al.* (2010). This variation is believed to be an adaptation to challenging environmental conditions (Thorpe, 2007).

Domesticated fish species such as the salmonids are subject to seasonal and local variations in the environment during the sea phase. The normal practice in the production cycle of farmed Atlantic salmon *Salmo salar* L. 1758 involves the transfer of in-season (S1) juveniles (smolts) from fresh water to sea water during the spring (April to June). The weeks following transfer to the sea are a period in which energy

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demands are high and the fish may have poor condition factor and low levels of muscle fat (Jobling *et al.*, 2002; Lysfjord *et al.*, 2004; Alne *et al.*, 2011). Additionally, outbreaks of various viral or bacterial diseases often occur during this period (Bowden *et al.*, 2002; Rørvik *et al.*, 2007; Hjeltnes, 2014). In contrast, the period from summer until late autumn is characterized by rapid growth and high fat deposition (Mørkøre & Rørvik, 2001; Alne *et al.*, 2011). The first winter at sea is a further energy-demanding period for *S. salar*, during which levels of muscle fat fall due to an increased energy demand for maintenance at the low sea temperatures (Lega *et al.*, 1992; Handeland *et al.*, 2000; Mørkøre & Rørvik, 2001). Thus, seasonal variations in temperature and day length affect growth and fat accumulation in this species (Mørkøre & Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2011). These factors, combined with disease outbreaks and internal energy status, significantly influence the production biology of farmed *S. salar*.

The depletion of energy stores after the first winter at sea may challenge the success of the reproductive strategy of *S. salar*. After the maturation process has been initiated during the winter season (Oppedal *et al.*, 1999, 2006), the availability of appropriate energy or fat reserves during the spring period is a major factor in the maturation process and low energy or fat levels arrest further progress (Rowe & Thorpe, 1990; Thorpe *et al.*, 1990; Kadri *et al.*, 1996; Duston & Saunders, 1999; Duncan *et al.*, 2002). Female *S. salar*, in particular, must invest more energy in the development of gonads than males (Aksnes *et al.*, 1986). Females normally mature later in life than males, most commonly in the autumn after two or more winters in the sea (Duston & Saunders, 1999).

The fatty acid tetradecylthioacetic acid (TTA) increases the number and the size of peroxisomes and mitochondria in mammals, which leads to an increased capacity for β-oxidation of fatty acids (Bremer, 2001). TTA also decreases the levels of plasma lipids and adipose lipid stores and enhances the transport of fatty acids to the liver (Berge *et al.*, 2002). In recent years, TTA has been of interest as an additive for fish feed (Moya-Falcón *et al.*, 2004; Kennedy *et al.*, 2007; Rørvik *et al.*, 2007; Alne *et al.*, 2009*a*, *b*; Arge *et al.*, 2012; Grammes *et al.*, 2012). In experiments when *S. salar* were given TTA-supplemented feed during the first spring in sea (April to June), it led to reduced fat content in white muscle. This is observed in both post-smolts (Alne *et al.*, 2009*a*) and larger individuals (Arge *et al.*, 2012). Early sexual maturation in male *S. salar* the following autumn (jack maturation) was significantly lower in the TTA-fed fish in both studies, possibly due to the lower accumulation of fat in the spring. Dietary TTA during the same time of year also found increased survival rates in *S. salar* post-smolts during natural outbreaks of infectious pancreas necrosis (Rørvik *et al.*, 2007) and heart and skeletal muscle inflammation (Alne *et al.*, 2009*b*). In both studies, the findings were related to a stimulation of energy metabolism by re-allocation of lipids from storage to energy production. Thus, the principal mechanism of TTA is to facilitate an increase in β-oxidation during energy-demanding periods.

As mentioned above, the energy status of *S. salar* in spring seems closely related to sexual maturation. This raises the question of whether fat dynamics may differ between males and females. TTA exerts at least part of its metabolic effects through the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors (Skrede *et al.*, 1997; Bremer, 2001) and it has recently been shown that crosstalk takes place between PPARs and oestrogen receptors in mice *Mus musculus* (Bang Hyun Kim *et al.*, 2009) and possibly also in brown trout *Salmo trutta* L. 1758 (Batista-Pinto *et al.*, 2009). To gain a better understanding of reproductive biology in *S. salar*, the hypothesis was that

inclusion of TTA in the diet may lead to sex-specific differences in fat–lipid accumulation. The hypothesis was tested during two periods of high energy demand: the first spring and the second winter in the sea.

MATERIALS AND METHODS

FISH AND REARING CONDITIONS

Experiments were performed at the former Nofima Marine research station at Ekkilsøy, on the west coast of Norway (63° N). The study was a part of a larger project that extended from sea transfer in April 2009 until May the following year. Three thousand in-season (S1) *S. salar* smolts from the Rauma strain (Rauma Broodstock AS; www.vikenco.no), were bulk weighed on the 15 April 2009 and distributed among six tanks (500 fish per tank) on a truck at the hatchery (Straumsnes Settefisk AS). The fish were transported to the research station and transferred to six net pens ($5 \times 5 \times 5$ m), one tank per pen. The mean body mass (M) of the fish was 105 g at sea transfer. One of two dietary treatments was allocated to each pen at random, in triplicate. The net pens were located at the same pier and exposed to the natural photoperiod throughout the experiment. The ambient seawater temperature was recorded at a depth of 3 m and was 6·9° C at sea transfer and averaged 8·7° C during the experiment. The maximum seawater temperature was 16·3° C, recorded in August 2009 and the minimum was 2·7° C, recorded in February 2010.

SAMPLING AND FISH ANALYSES

Before sea transfer, 30 (3×10) fish of the total start population were sampled, representing the overall mean mass. Thereafter, 10 fish from each net pen, representing the overall mean mass of the respective pen were sampled on five occasions: 6 weeks (May 2009), 10 weeks (June 2009), 15 weeks (July 2009), 22 weeks (September 2009) and 34 weeks (December 2009) after sea transfer. The final sampling was carried out when the experiment was terminated (on 18 May 2010). The fish were weighed in bulk on each sampling occasion. The sampled fish were anaesthetized with MS-222 (Alpharma; www.alpharma.com) and killed by a sharp cranial blow. M and fork length (L_F) of each of the sampled fish were recorded. Sexual maturation was detected by visual observation of secondary sexual characteristics such as body colour and by examination of gonads. Sex was determined by visual inspection of the gonads. The fillet from the Norwegian Quality Cut (NQC) (NS 9401, 1994) of each sampled fish was photographed and the % fat in the cutlet was determined by digital image analysis using the PhotoFish system (Folkestad *et al.*, 2008). A total of 360 fish were sampled, of which 50·8% were females and 49·2% were males.

DIETARY TREATMENTS AND FEEDING

The diets were based on extruded fishmeal-based pellets (3, 4·5, 7 and 9 mm) commercially produced by Skretting AS (www.skretting.no). The experimental diets were obtained by coating the commercial pellets in a blender. The control diet was used both for the TTA study presented here and other experimental diets in a more extensive study and thus all diets were prepared in 25 kg batches by first coating the pellets with distilled water (0·9 l) and leaving them to dry for 1 day. Pellets intended for use in the TTA study were then coated with TTA dissolved in heated rapeseed oil (0·5 l at 70° C) to an inclusion level of 0·25% TTA (w/w). The rapeseed oil was heated to dissolve the TTA and the control diet was also coated with the same amount of heated and un-supplemented rapeseed oil.

TTA was administered over two periods. The first period lasted *c.* 10 weeks, from the start of the experiment at the end of April until 2‰ (w/w) TTA of the initial total biomass (mean M 105 g) had been eaten in each respective net pen. The second period was of duration 6 weeks from 16 January 2009 to 26 February 2009 and during this period 0·4‰ of the initial total biomass (mean M 2085 g) was eaten. The reduction in the second dosage was made on the basis

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TABLE I. Chemical composition of the control diet at the different pellet sizes fed to *Salmo salar*

Pellet size (mm)	3	4·5	7	9
Dry matter ($g\,kg^{-1}$)	912	898	889	927
Moisture ($g\,kg^{-1}$)	88	102	111	73
In dry matter:				
Crude lipid ($g\,kg^{-1}$)	250·8	289·2	332·5	350·4
Crude protein ($g\,kg^{-1}$)	468·8	421·2	386·7	352·3
Ash ($g\,kg^{-1}$)	65·7	73·6	49·8	53·8
Starch ($g\,kg^{-1}$)	53·8	50·3	72·9	76·0
NSP ($g\,kg^{-1}$)	73·0	63·8	47·1	94·6
Energy ($MJ\,kg^{-1}$)	23·0	23·3	24·2	25·1
Astaxanthin ($mg\,kg^{-1}$)	57·5	49·4	40·0	36·2

NSP, non-starch polysaccharides, calculated as: 1000 − (crude lipid + crude protein + ash + starch + moisture).

of previous knowledge of an increased effect of TTA at colder temperatures (Gjøen *et al.*, 2007). The TTA diet was replaced by the control diet after each period. The pellet size was adjusted to fish size in accordance with the feed manufacturer's guidelines. The fish were fed by automatic feeders in excess of the recorded feed intake, four times per day. Waste feed was collected after each feeding period and pumped up into wire mesh strainers (Einen *et al.*, 1999). The amount of dry matter recovered under the present conditions on the site was determined for each feed and a correction applied for the loss of dry matter in uneaten feed (Helland *et al.*, 1996).

CHEMICAL ANALYSES OF FEED

The control diet was analysed for dry matter, ash, crude protein, crude lipids, starch and energy (Table I). Dry matter was determined gravimetrically by drying at 105° C to constant mass. Ash was determined by flame combustion and heating to 550° C until constant mass. The crude protein was analysed as nitrogen × 6·25, using the automated Kjeldahl method (Tecator; www.foss.us). Crude lipid was analysed by HCl hydrolysis and extraction in petroleum ether, using the SOXTEC HT 6 system and a SOXTEC1047 hydrolysing unit (Tecator). Starch was analysed as glucose after enzymatic hydrolysis, using a Megazyme K-TSTA 05/06 total starch assay kit (www.megazyme.com). The energy content was determined by using a Parr 1271 bomb calorimeter (Parr Instruments Co.; www.parrinst.com). The feed formulation and amino acid composition of the 9 mm pellet size of the control diet is presented in Larsson *et al.* (2014).

Analyses of TTA content were carried out as described in Kennedy *et al.* (2007) at the Institute of Medicine, Haukeland University Hospital, Bergen, Norway. The inclusion level of TTA in the diets was 0·23%, which was 0·02% below the intended inclusion level. A leakage test was done to measure the loss of TTA from the pellets into water. The leakage test involved placing 10 g of pelleted diet and 45 ml of water into a 250 ml glass bottle and shaking gently by hand for 15 s. The pellets were subsequently transferred to a new 250 ml glass bottle and the procedure repeated. The total time that the pellets were in water (including transfer time) was 3 min. The test showed that *c.* 9% of the TTA leaked from the pellets during this time. In the actual experiment, however, the pellets were eaten by the fish in a matter of seconds, considering the small size of the net pens and the amounts of uneaten feed collected.

CALCULATIONS

The growth rate of the fish was measured as the thermal-unit growth coefficient (C_{TG}) (Cho, 1992). C_{TG} incorporates both fish size and temperature and was calculated as:

$1000\left(M_{d1}^{1/3}-M_{d0}^{1/3}\right)(\Sigma T)^{-1}$, where M_{d1} is the final body mass (g), M_{d0} is the initial body mass (g) and ΣT is the sum of day-degrees during the period. Feed conversion ratio (R_{FC}) was calculated as: $(B_1 - B_0)$ $[1000\ (M_{d1} - M_{d0} + M_{dead})]^{-1}$, where B_0 is the initial biomass and B_1 is the final biomass of food and M_{dead} is the total mass of fish that died during the experiment. The specific feeding ratio (R_{SF}) was calculated as: {kg feed supplied $[(B_0 + B_I)^{-2})^{-1}] \times 100$}, where B_0 is the initial biomass, B_1 is the final biomass. The condition factor (K), which relates the mass of the fish to its length, was calculated as: $K = ML_F^{-3}100$, where M is body mass (g) and L_S is standard length (cm).

STATISTICAL ANALYSIS

Net pen was used as the experimental unit. C_{TG}, R_{FC}, R_{SF}, M, K and amount of muscle fat were used as response variables. Diet, sex and sampling point were used as class variables. A full model including both single variables and interactions between variables was applied in which the data were statistically tested for sex-specific effects using the SAS software package (SAS Institute Inc. 1990; www.sas.com): $Y_{ijl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijl}$, where Y_{ijl} is the response variable, μ an overall effect, α_i the main effect of diet i, β_j the main effect of sex j, $(\alpha\beta)_{ij}$ an interaction effect, unique to the particular combination of levels and ε_{ijl} is a random error.

r^2 expresses the proportion of the variance explained by the model and was calculated as: between-group sum of squares divided by the total sum of squares (type III).

Significant differences between means were ranked by Duncan's multiple-range tests. The significance level was set at $P \leq 0{\cdot}05$ and $P \leq 0{\cdot}10$ was considered to be a trend. The results are presented as means ± S.E., unless otherwise stated.

RESULTS

PRODUCTION DATA

There were no significant differences in M or mortality between the groups given the two dietary treatments. At stocking in April, the average M of the fish was 105 g. At the final sampling and termination of the experiment in May the following year, the overall M was 3009 ± 45 g. Similarly, the daily mass increase, C_{TG}, R_{SF} and R_{FC} did not differ significantly between the fish groups throughout the experimental period. Table II presents the pooled production data.

SEASONAL VARIATION IN CONDITION FACTOR (K)

K of all fish varied with season. The mean K at sea transfer was 1·29 and it declined markedly in both dietary groups during the first 10 weeks in the sea (April to June). It increased during the early autumn and then levelled off. The K did not fall during the winter period (Fig. 1).

MUSCLE-FAT CONTENT

The mean muscle-fat content of the post-smolt groups after the first period of being fed TTA (the first spring in the sea) was 4·1 ± 0·3%. The muscle-fat content increased throughout the late summer and autumn, as did the K and M. The mean muscle-fat content was 17·8 ± 0·1% for the control group at the final sampling in May and 16·4 ± 0·1% for the TTA group.

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TABLE II. Overall mean body mass in S1 *Salmo salar* at each sampling, and periodic mean temperature (° C), daily mass increase, thermal-unit growth coefficient (C_{TG}), specific feeding rate (R_{SF}) and feed conversion ratio (R_{FC}) ($n = 6$)

	Sampling month					
	May	June	July	September	December	May
Body mass mean ± S.E. (g)	166 ± 4	234 ± 3	414 ± 3	846 ± 17	1775 ± 31	3009 ± 45
	April to May	May to June	June to July	July to September	September to December	December to May
Temperature mean ± S.D. (° C)	9·2 ± 1·6	12·1 ± 0·6	11·5 ± 1·5	15·2 ± 0·7	10·1 ± 1·8	4·7 ± 1·3
Daily mean ± S.E. mass increase (g day^{-1})	1·6 ± 0·1	2·5 ± 0·1	5·3 ± 0·1	9·0 ± 0·3	11·6 ± 0·2	7·9 ± 0·1
C_{TG} mean ± S.E.	2·19 ± 0·10	2·04 ± 0·07	3·31 ± 0·07	2·75 ± 0·08	3·29 ± 0·02	3·15 ± 0·04
R_{SF} mean ± S.E.	0·75 ± 0·03	0·94 ± 0·03	1·23 ± 0·03	1·30 ± 0·05	0·82 ± 0·01	0·36 ± 0·00
R_{FC} mean ± S.E.	0·64 ± 0·01	0·76 ± 0·01	0·75 ± 0·00	0·94 ± 0·01	0·94 ± 0·01	1·12 ± 0·01

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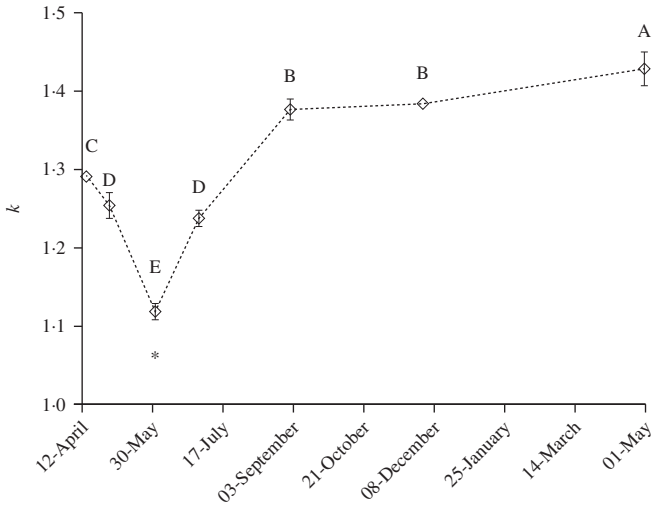

FIG. 1. Development of the overall mean ± S.E. ($n = 6$) condition factor (K) in S1 *Salmo salar* fed dietary tetradecylthioacetic acid (TTA) during the experimental period from April to May in the following year. Different upper case letters show significant differences between sampling points. *, sampling points after the two periods of TTA administration.

When applying the full statistical model, the class variable sex was not significant with respect to muscle-fat content, for either of the periods of TTA administration. Sex as a single class variable was therefore excluded from the model. This gave a reduced model based on diet and the combined effect of diet and sex was significant in both the first and second spring periods in the sea. This also explained the variation in muscle fat most convincingly (Table III). There was a significant interaction between the dietary treatment and sex during the first spring, while the interaction during the second spring was borderline significant ($P < 0{\cdot}10$). This made it necessary to test the statistical effects of diet within each sex separately.

The effect of diet on muscle-fat content during the first spring in the sea was significant for females but not for males (Table III and Fig. 2). The effect of diet on muscle-fat

TABLE III. Overview of probability (P-value) and the total variation explained (r^2) by the models used in statistical analyses of muscle-fat content (%) in S1 *Salmo salar* in the first and second spring in the sea

		First spring		Second spring	
Class variables		P	r^2	P	r^2
Diet + diet × sex	Full significant statistical model	<0·05	0·69	<0·001	0·86
Diet	Single variable within the model	<0·05	0·33	<0·001	0·72
Diet × sex	Interaction term within the model	<0·05	0·36	<0·10	0·13
Diet	Between males	>0·10	0·04	<0·01	0·90
	Between females	<0·01	0·86	<0·05	0·72

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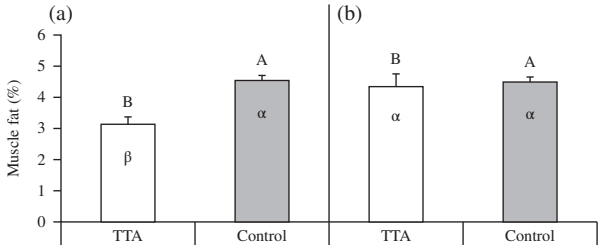

FIG. 2. Mean + S.E. ($n = 3$) muscle-fat content of (a) female and (b) male S1 *Salmo salar* given (□) a diet supplemented with tetradecylthioacetic acid (TTA) or (■) a non-supplemented control diet during the first spring in the sea. Significant differences between diets within a sex are indicated by different letters on top of the bars; a general test for significant differences in the means across both sexes is indicated by different letters inside the bars.

content during the second spring in the sea was significant in both sexes (Table III and Fig. 3). The fat content of muscle for females fed the TTA diet was significantly lower than that of all other groups during the first spring in the sea (Fig. 2). Both female and male fish fed the TTA diet had significantly lower levels of muscle fat during the second spring in the sea than fish fed the control diet. In contrast to the previous spring, however, the level of muscle fat in the males in the TTA group was significantly lower than it was in the females (Fig. 3).

CONDITION FACTOR *K*

K interacted in a significant manner with both diet and sex in both the first ($P < 0{\cdot}05$, $r^2 = 0{\cdot}6$) and the second spring ($P < 0{\cdot}01$, $r^2 = 0{\cdot}78$) in the sea. The effect on *K* was significant for females during the first spring in the sea, but not for males. *K* of females fed the TTA diet was significantly lower than *K* of all other groups (Table IV). This is the same pattern as that seen for muscle fat. The pattern for *K* was again similar to that of muscle fat during the second spring in the sea, during which diet had an effect on the *K* for males and almost so for females ($P < 0{\cdot}10$). *K* of males was thus significantly lower than that of females treated with dietary TTA (Table IV).

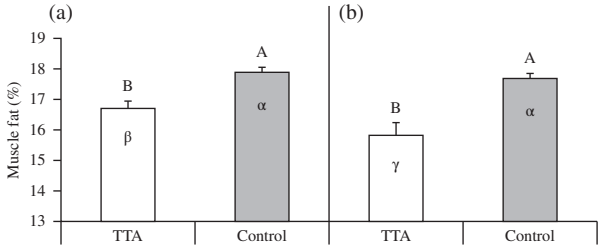

FIG. 3. Mean + S.E. ($n = 3$) muscle-fat content of (a) female and (b) male S1 *Salmo salar* during the second spring in the sea given (□) a diet supplemented with tetradecylthioacetic acid (TTA) or (■) a non-supplemented control diet during the first winter in the sea. Significant differences between diets within a sex are indicated by different letters on top of the bars. A general test for significant differences in the means across both sexes is indicated by different letters inside the bars.

TABLE IV. Condition factor (*K*) in S1 *Salmo salar* males and females given a diet supplemented with tetradecylthioacetic acid (TTA) or a control diet during the first spring and winter in the sea. *P*-values show statistics of the dietary treatments within sex. Different superscript letters indicate significant differences over both sexes ($P < 0{\cdot}05$) across rows. All values are mean ± S.E. ($n = 3$)

	TTA-supplemented diet *K*		Control diet *K*		Diet within sex *P*	
Sampling	Males	Females	Males	Females	Between males	Between females
First spring	$1{\cdot}14 \pm 0{\cdot}02^{a}$	$1{\cdot}07 \pm 0{\cdot}01^{b}$	$1{\cdot}13 \pm 0{\cdot}02^{a}$	$1{\cdot}14 \pm 0{\cdot}01^{a}$	>0·10	<0·01
Second spring	$1{\cdot}33 \pm 0{\cdot}02^{c}$	$1{\cdot}41 \pm 0{\cdot}01^{b}$	$1{\cdot}43 \pm 0{\cdot}02^{b}$	$1{\cdot}52 \pm 0{\cdot}04^{a}$	<0·05	<0·10

BODY MASS OF THE SAMPLED FISH

Neither diet nor sex affected *M* of the sampled fish during the first or second spring. Therefore, there was no statistically significant difference in *M* between males and females sampled for analysis in the two dietary groups at the two spring samplings.

SEXUAL MATURATION

Only 12 fish, six in each of the two dietary groups, were in the process of sexual maturation at the sampling occasion in September. All fish that were undergoing sexual maturation were males.

DISCUSSION

This study demonstrated a significant dietary effect of TTA and an interaction between diet and sex in *S. salar* during the first spring and winter in the sea. This resulted in significantly different levels of muscle fat in males and females during the following spring. The validity of these observations is supported by corresponding changes in *K*. The results suggest that the reproduction biology of *S. salar* seems related to, among other factors, different dynamics of fat accumulation in the sexes.

TTA administered during the first spring reduces the level of muscle fat in S1 *S. salar* smolts, as reported by Alne *et al.* (2009*a*). The current study has shown, however, that *S. salar* males and females respond differently to the TTA treatments during both the first spring and the winter in the sea. Batista-Pinto *et al.* (2009) showed that PPAR*α* mRNA expression was significantly higher in female *S. trutta* in May than in the rest of the year, whereas the level in males did not change. The authors suggested that the PPAR*α* in *S. trutta* females was under oestradiol modulation and further, that cross-talk between this and the oestrogen receptor may occur. If this is also holds for *S. salar*, it is conceivable that this mechanism influenced the effect of TTA on females in this study, but this needs more investigation.

Alne *et al.* (2009*a*) administered TTA in the feed for a short period after sea transfer and found reduced incidence of early sexual maturation in the following autumn. The authors related this to a reduction of fat in the TTA-treated males during the spring. Consequently, it was surprising to see a significant fat reduction only in females in

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the current study. Very few fish, however, reached early maturation in the autumn and the number that did so was the same in the TTA group as in the control group. This indicates a lower effect of TTA than in Alne *et al.* (2009*a*), probably due to the lower TTA-inclusion level (0·25 *v*. 0·50%) in this study. Another consideration is that sexual maturation processes in *S. salar* males may differ with age and size (Taranger *et al.*, 2010).

The lower dose in the winter period was sufficient to provoke an effect of TTA. The reduction in muscle fat during this period was, in addition, significantly greater in males than females. The reason for the differences in response to TTA between sexes at the different time periods is not clear, but may have involved endogenous mechanisms as discussed above. The effects of TTA have been extensively described in male mammals (Frøyland *et al.*, 1995; Bjørndal *et al.*, 2013) and to a lesser extent in females (Stunes *et al.*, 2011; Skrede *et al.*, 2012). As far as is known, this study is the first direct comparison.

The most marked differences in response to TTA occurred during the first spring in sea. Early maturation in males takes place during the spring of the same year as sea transfer (jacks, Duncan *et al.*, 2002; Alne *et al.*, 2009*a*). Thus, the drive for increased development in the gonads may be very persistent in a large proportion of S1 males and it may be important to maintain a high body energy level. This mechanism may have counteracted the TTA treatment in males at this time. Females at this developmental status, in contrast, may not have reached a sufficient energy level for maturation and may therefore respond to TTA by mobilising fat for energy, as seen in this study. Lipid levels are significantly higher in both male and female *S. salar* after 1 year in the sea and both sexes may be anticipated to mature to a certain extent during the second spring and into the next autumn. Thus, in this regard, a more similar response to TTA may perhaps be expected during the second, rather than the first, spring in the sea. Batista-Pinto *et al.* (2009) have shown that seasonal and sexual differences in the expression of mRNA for PPARα occur in salmonids and Costet *et al.* (1998) showed that PPARα is involved in lipid homeostasis in mice, in which the control of circulating lipids, fat storage and obesity are all sexually dimorphic. Thus, if the same mechanisms exist in *S. salar*, it is possible that the different treatment responses were a result of an interrelationship between TTA, sex and seasonal differences in PPAR expression.

The transfer to sea water in spring affects the general performance of S1 *S. salar* and leads to reduced growth, lower *K* and lower whole body fat levels (Mørkøre & Rørvik, 2001; Alne *et al.*, 2011). Fat reserves and *K* in salmonids are often closely related (Herbinger & Friars, 1991; Alne *et al.*, 2011) and *K* may thus be an indicator of muscle-fat content as seen in this study.

In an ecological perspective, much knowledge has accumulated concerning factors affecting general growth and survival of wild *S. salar* in the marine phase (Windsor *et al.*, 2012). Less is known, however, about the factors affecting specifically the females during the time at sea. The sex specific effects of altered muscle-fat accumulation demonstrated in this study during time of spring, may therefore partly explain the variable male–female ratios seen in returning *S. salar* to their native rivers (Fjallstein, 1989; Guðjónsson *et al.*, 1995).

In conclusion, sex-specific differences in the level of muscle fat occurred in *S. salar* when TTA was fed during the first and second spring in the sea. The effect was greater when the TTA was administered during the first spring. The relationship between fat accumulation and sexual maturation in *S. salar* males and females may be better

understood from experiments in which TTA is used as an experimental feed additive. The results demonstrate the importance of considering both sex and season when studying fat dynamics and reproduction biology in *S. salar*.

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Paper III

Paper III

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Effects of tetradecylthioacetic acid (TTA) treatment on lipid metabolism in salmon hearts—in vitro and in vivo studies

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Abstract In intensive farming of Atlantic salmon, a large proportion of observed mortality is related to cardiovascular diseases and circulatory failure, indicating insufficient robustness and inadequate cardiac performance. This paper reports on the use of tetradecylthioacetic acid (TTA) where the main objective was to enhance utilisation of fatty acids (FA), considered the main energy source of the heart. In this study, three experiments were conducted: (I) an in vivo study where salmon post-smolt were administrated dietary TTA in sea, (II) an in vitro study where isolated salmon heart cells were pre-stimulated with increasing doses of TTA and (III) an in vivo experiment where salmon post-smolt were subjected to injections with increasing doses of TTA. In study I, TTA-treated fish had a smaller decrease in heart weight relative to fish bodyweight (CSI) in a period after sea transfer compared to the control. This coincided with lowered condition factor and muscle fat in the TTA-treated fish, which may indicate a higher oxidation of lipids for energy. In study II, the isolated hearts treated with the highest dose of TTA had higher uptake of radiolabelled FA and formation of CO_2 and acid-soluble products. In study III, expression of genes regulating peroxisomal FA oxidation, cell growth, elongation and desaturation were upregulated in the heart of TTA injected salmon. In contrast, genes involved in FA transport into the mitochondria were not influenced. In conclusion, these experiments indicate that TTA enhances energy production in salmon hearts by stimulation of FA oxidation.

Keywords Atlantic salmon · Heart · Fatty acid metabolism · TTA

Introduction

In salmonids, like the Atlantic salmon (*Salmo salar* L.), cardiac performance or insufficient oxygen distribution capacity has been related to increased mortality in commercial fish farms. Lower tolerance to transportation and handling, adaptation ability towards environmental changes and increased physical demands have been reported (Poppe et al. 2003; McClelland et al. 2005; VKM 2014). To counteract suboptimal cardiac performance, Castro et al. (2011) showed that physical training of young salmon stimulated cardiac growth and led to higher disease resistance and better growth in general. Such training schemes may, however, be difficult to implement on a large scale in commercial salmon farms and other approaches towards higher robustness may be of interest.

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Utilisation of fatty acids highly dominates energy metabolism in high-performance fish (Patton et al. 1975; Moyes et al. 1992; West et al. 1993; Castro et al. 2013), and to promote rapid growth in farmed salmon, commercial feeds normally contain high fat levels. This may, however, lead to undesired fat deposition around the heart, arteriosclerosis and other life-style-associated diseases similar to what is seen in mammals (Poppe and Taksdal 2000; Brocklebank and Raverty 2002). Hence, ways to facilitate optimal utilisation of dietary fat and not excessive storage ought to be sought. As such, the fatty acid *tetradecylthioacetic acid* (TTA) has been tested on salmon as a feed additive. TTA is a synthetic 16 carbon saturated fatty acid, with a sulphur substitution in the β-position which inhibits normal β-oxidation of this fatty acid (Skrede et al. 1997). TTA can be catabolised through ω/β-oxidation and then via sulphur oxidation, albeit at slow rates (Skrede et al. 1997). The biological effect of TTA is especially through its action as an agonist for PPARs (peroxisome proliferator-activated receptors) and thus on the molecular level, increases fatty acid catabolism and decreases plasma lipids, adipose lipid stores and transportation of fatty acids (Berge et al. 1989, 2002; Hvattum et al. 1993; Moya-Falcon et al. 2004; Kennedy et al. 2007; Rørvik et al. 2007; Alne et al. 2009 and Grammes et al. 2012a, b). Additionally, TTA has been shown to increase both number and size of peroxisomes and mitochondria in mammals, which in turn increases cell β-oxidation capacity (Bremer 2001). In periods of high energy demand for salmon, testing of TTA of a more productional or strategic character has been done: Alne et al. (2009) and Arge et al. (2012) reduced body fat stores in salmon in the first spring at sea by supplementing TTA in the diets. The treatments resulted in lower incidence of early male sexual maturation the following autumn. Rørvik et al. (2007) and Alne et al. (2009) observed reduced mortalities in salmon during outbreaks of heart and skeletal muscle inflammation (HSMI) as well as infectious pancreas necrosis (IPN), and the authors pointed at mobilisation and increase of available energy resources as possible reasons. Dessen et al. (2016) further reported that salmon males and females responded differently to TTA first spring and first winter in sea and related this to different fat accumulation progression between the sexes depending on the time of year and body size.

This paper describes three separate experiments: two studies in vivo (*I and II*) and one heart cell study in vitro. The purpose of the small-scale in vivo *I* experiment was to test the general effect of TTA supplementation in feed for salmon post-smolts in the weeks after transfer to seawater. Based on the results of the in vivo *I* experiment, the objective of the in vitro experiment was to pre-stimulate salmon heart cells in culture with increasing doses of TTA and to study the response in fatty acid uptake and β-oxidation in absence of endogenous or systemic factors. Unfortunately, after two rounds of testing, it was not possible to detect any significant changes on the genetic level in the cell cultures. Thus, based on the knowledge gained from the two previous experiments, the purpose of the second experiment in vivo (*II*) was by injections of TTA, to further elucidate possible effects on genes involved in heart fatty acid metabolism and cell growth.

Methods

In vivo study I

The experiment was done at the former Nofima Marin research station at Ekkilsøy, on the west coast of Norway (63° N). The study was an integrated part of a larger experiment that lasted from sea transfer in April 2009 until May 2010 (see Dessen et al. 2016). Three thousand in-season Atlantic salmon smolts with a mean body weight of 105 g were distributed among six net-pens (500 fish per pen) on 15 April 2009. Three net-pens were fed a commercial extruded diet (3-mm pellet; crude protein, 514 g kg^{-1}; crude lipids, 275 g kg^{-1}; crude energy, 25.2 MJ kg^{-1}) with an inclusion level of 0.25% TTA (*w*/*w*), and three net-pens were fed the same commercial diet without inclusion of TTA. The TTA diet was fed from 15 April to 24 June. The pens were located at the same pier (randomised block design) and exposed to ambient seawater temperature and natural photoperiod. The part of the study reported here lasted from 15 April until 29 July 2009. The average temperature during the study was 10.7 °C. At the start of the experiment, 10 fish were sampled to determine the initial cardio-somatic index (CSI) and condition factor (CF). Three samplings were conducted during the trial: 27 May, 24 June and 29 July 2009. At each sampling, all fish were anaesthetized (MS-222 metacaine 0.1 g L^{-1}, Alpharma, Animal Health, Hampshire, UK) and bulked weighted. All fish were starved for 2 days prior to the samplings. At each sampling, 10 fish from each pen

were collected. The mean weight of the sampled fish represented the mean body weight of the fish in the pen, which was obtained from bulk weighing at each sampling point. The sampled fish were killed by a blow to the head before the gill arches were cut, and the fish were bled out in ice water. Fork length and bodyweight of each individual fish were recorded again after bleeding. The fish were opened and sex determined by visual inspection of the gonads. The heart was removed and weighted to calculate the CSI. At each sampling point, the Norwegian Quality Cut, NQC (NS9401, 1994) from the left fillet was analysed for fat content as described in Dessen et al. (2016). The organ index (CSI) was calculated as Y (g) × body weight (g)$^{-1}$ × 100, where Y is the weight of the measured heart. The condition factor was defined as 100 × body weight (g) × fork length^{-3}. For more details about the preparation of dietary treatments, experimental design and the fish material, see Dessen et al. (2016).

In vitro study

Materials

Tetradecylthioacetic acid was obtained from Sigma-Aldrich (MO, USA). Isotope-labelled [1-^{14} C] palmitic acid (40–60 mCi (1.48–2.22 GBq)/mmol) was obtained from PerkinElmer (Waltham, MA). Collagenase TYPE 1 (267 U/mg) was obtained from Laborell (Worthington), collagenase 740 U/mg, heparin, laminin, albumin was obtained from Sigma-Aldrich. FBS (foetal bovine serum) was obtained from PAA Laboratories GmbH, Pasching, Austria. Buffering agent 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Leibowitz's L-15 media (GlutaMAX™), phosphate buffer saline (PBS), ethylenediaminetetra-acetic acid (EDTA) perfusion solution and antibiotic-antimycotic stabilised solution was obtained from Sigma-Aldrich.

Experimental fish and isolation of cardiomyocytes

Atlantic salmon (10 fish in total) of approximately 500 g (NINA, Solbergstrand, Norway) had been reared in indoor seawater tanks at constant 8 °C and kept on a long-day photoperiod by supplying 24-h artificial light. The fish had been given a standard commercial diet prior to isolation of cardiomyocytes. The fish were anaesthetized in Metacain (MS-222, 0.1 g L^{-1}) to death. To prevent blood clotting, 0.1 mL heparin (5000 U/mL) was injected into the dorsal vein before the abdomen was opened. The intact hearts were carefully excised and quickly transferred to sterile petri dishes. The bulbus arteriosus was cannulated and with a peristaltic pump, the heart was perfused (4 mL/min) following a two-step collagenase procedure developed by Seglen (1976) and modified by Dannevig and Berg (1985). Firstly, heart was perfused with a buffer containing in mM: 100NaCl, 10 KCl, 1.2 KH2PO4, 20 glucose, 10 Hepes sodium salt, 10 BDM (C4H7NO2), 4 MgSO4 and 50 taurine (Nurmi and Vornanen 2002), for 5 min to flush out the blood and open the tight junctions. Thereafter followed a 20 min perfusion applying the same buffer + 0.75 mg/mL of the protease collagenase type 1 and 0.5 mg/mL trypsin. Cardiomyocytes were subsequently isolated by gentle shaking of the digested heart in Leibowitz's L-15 medium. The suspension of cells obtained in this manner was filtered through a 100-μm nylon filter. Cardiomyocytes were washed three times in Leibowitz's L-15 medium and sedimented by centrifugation for 10 min at 1250 rpm at 4 °C. The cardiomyocytes were re-suspended in growth media containing Leibowitz's L-15 media with FBS (10%, PAA Laboratories, Australia), Penicillin-Streptomycin solution (1%, PAA Laboratories, Australia) and Hepes (10 mM, Sigma-Aldrich). Cell viability was assessed by staining with Trypan Blue (0.4%, Sigma-Aldrich). Mean yield was approximately 2.1 × 10^6 cardiomyocytes in 24 mL and were plated onto cell culture flasks coated with laminin (1.2 μL/cm^2, Merck, Darmstadt, Germany), and left to attach overnight at 13 °C.

Enrichment of cardiomyocytes with TTA

The cultivated cardiomyocytes were washed twice with L-15 medium without serum supplementation, and then incubated with TTA. The TTA was added to the growth media (containing 2% FBS) in the form of sodium salts bound to BSA (2.7/1, molar ratio). Briefly, 5 mg TTA was dissolved in preheated 0.1 M NaOH (0.70 mL). The FA-NaOH solution was then transferred to 2.2 mL PBS-albumin, which contained 0.43 g albumin. The pH was adjusted to 7. The solution was made as a stock solution of 6 mM. The cell culture media were supplemented with TTA in the following concentrations: 0 μM (control), 30, 60 and 120 μM. The cells were incubated in triplicates for 3 days at 13 °C with TTA.

Incubation of cells with radiolabelled 16:0

After the pre-incubation period where the cells had been enriched with TTA, isotope-labelled [1-^{14}C] palmitic acid (PA) was added to the growth medium in order to study the effect of endogenous TTA on the metabolism of the radiolabelled FA substrate in the cardiomyocytes.

The cultivated cardiomyocytes were first washed with L-15 medium without serum supplementation, and then incubated for 36 h with 1200 nmol [1-^{14}C] 16:0 (final concentration of 20 μM) in a total volume of 5 mL of L-15 culture medium with 2% FBS. The specific radioactivity of the FA was 50 mCi/mmol (1.8 μCi of radioactive FA substrate was added to each cell flask). The radiolabelled FA was added to the medium in the form of its sodium salt bound to FA-free bovine serum albumin (BSA) (the molar ratio of FA to BSA was 2.7:1). After incubation, the culture medium was transferred from the culture flasks to vials and centrifuged for 5 min at 50×*g*. The supernatants (culture media) were immediately frozen at − 80 °C and stored for determination of un-metabolised radiolabelled substrate and oxidation products. Cardiomyocytes supplemented with 16:0 were washed twice in PBS that contained 1% albumin, and once more with regular PBS. The cells were then harvested in 2 mL of PBS and stored at − 80 °C before the radiolabelled lipid classes were analysed.

Prior to incubation, aliquots of 10, 20, 30, 40 and 50 μL of the incubation medium with the radioactive 16:0 were transferred into different vials with 8 mL of Ecoscint A scintillation liquid in order to count total radioactivity and the specific radioactivity (cpm/nmol FA) was subsequently calculated. The samples were counted in a scintillation counter TRI-CARB 1900 TR (Packard Instrument Co., IL, USA).

Lipid extraction and analysis of lipid classes

Total lipids were extracted from cells incubated with radiolabelled 16:0 as described by (Folch et al. 1957). The chloroform phase was dried under nitrogen gas, and the residual lipid extract was redissolved in 1 mL of chloroform. Fifty microliters of chloroform was transferred into vials containing 8 mL scintillation fluid for scintillation counting, and the rest was used for lipid analysis. Free fatty acids (FFA), phospholipids (PL), monoacylglycerols (MG), diacylglycerols (DAG) and triacylglycerol (TAG) were separated by thin-layer chromatography (TLC) using a mixture of petroleum ether, diethyl ether and acetic acid (113:20:2 *v*/*v*/*v*) as the mobile phase. The samples were applied onto silica gel TLC plates. The lipids were identified by comparison with known standards by a Bioscan AR-2000 Radio-TLC & Imaging Scanner and quantified with the WinScan Application Version 3.12 (Bioscan Inc., Washington, DC, USA).

Beta-oxidation

The capacity of β-oxidation of 16:0 was measured by determination of oxidation products (counting ^{14}C-labelled acid-soluble products (ASPs) and the $^{14}CO_2$ formed) essentially as described by Christiansen et al. (1976). The amount of gaseous [1-^{14}C] CO_2 produced during the incubation was determined by transferring 1.5 mL of medium to a glass vial, which was then sealed. The glass vial had a central well containing Whatman filter paper (diam. 125 mm) moistened with 0.3 mL of phenylethylamine/methanol (1:1, *v*/*v*). The medium was acidified with 0.3 mL 1 M $HClO_4$. The samples were incubated for 1 h, and then the wells, containing the filter papers, were placed into vials for scintillation counting.

The quantities of [1-^{14}C] ASP present were determined by acidifying 1 mL of the medium with 0.5 mL ice-cold 2 M $HClO_4$ and incubating the sample for 60 min at 4 °C. The medium was then centrifuged, and an aliquot of the supernatant was collected for scintillation counting.

HPLC separation of oxidation products in ASP

The remaining ASP supernatant was neutralised with NaOH, and the different ASPs were detected by using high-pressure liquid chromatography equipped with a ChromSep Inertsil C8-3 column (250 × 4.6 mm stainless steel), a UV detector at 210 nm and radioactive detector A-100 (Radiomatic Instrument & Chemicals, Tampa, FL, USA) coupled to the UV detector. The mobile phase was 0.1 M ammonium dihydrogenphosphate adjusted with phosphoric acid to pH 2.5, and the flow rate was 1 mL/min. The components were identified by comparison to external standards and retention times.

Protein measurements

The protein content of the cells was determined by using the total protein kit (Micro Lowry/Peterson's modification) (Peterson 1977, Lowry et al. 1951) and measured at 540 nm in a 96-well plate reader Titertek, Multiscan (Labsystem, Finland).

In vivo study II

Fish and fish treatment

This experiment was done at the Fiskaaling PF marine research station at Nesvík, Faroe Islands (62° N). Four weeks prior to the experiment, salmon post-smolts had adapted to full seawater in a 20-m^3 outdoor tank and were kept on a long-day photoperiod by supplying 24-h artificial light. Three days before the experiment, the fish (90.5 ± 0.7 g) were transferred to six 500-L indoor tanks (10 fish per tank). The fish were still kept on a long-day photoperiod and at ambient temperature (5.9 ± 0.1 °C) for 8 days. Oxygen was kept above 7 mg L^{-1} measured in the tank outlet. Feed, Havsbrún Margæti 3.0 mm (Havsbrún PF, www.havsbrun.fo), was offered continuously in excess by automatic feeders. Approximate feed composition was crude protein 48 %, fat 26% whereof the ratio of fish oil and rapeseed oil was about 60/40.

On day 1 of the experiment, all fish (10 fish/treatment) were anaesthetized (benzocaine 0.1 g L^{-1}, prepared at Tjaldurs Apotek, Faroe Islands) and given a 0.3-mL injection containing TTA into the muscle alongside the dorsal fin. TTA for injections was prepared by first dissolving 5 mg TTA in preheated 0.1 M NaOH (0.70 mL). The FA-NaOH solution was then transferred to 2.2 mL PBS-albumin, which contained 0.43 g albumin. The pH was adjusted to 7. The solution was made as a stock solution of 6 mM. Based on the results from the in vitro study, the injected treatment doses of TTA were chosen to be 58, 115, 231 and 461 μg/kg. The doses were prepared in physiological saline, and total injection volume corresponded to approx. 12% of total fish blood volume (Hjeltnes et al. 1992). The control fish were injected with physiological saline only. The fish were starved on day 8 (end of the experiment), and all fish were anaesthetized to death (benzocaine) and weight, fork length and sex recorded. Heart ventricle samples were collected and kept in RNA-later® (Thermo Fisher Scientific, thermofisher.com) and frozen at − 80 °C for later analyses.

RNA extraction and real-time PCR

RNA from heart ventricles was extracted using PureLink® RNA Mini Kit according to manufacturer's instructions. On-column PureLink® DNase (Thermo Fisher Scientific) was used to remove traces of DNA in the samples. Quantification and evaluation of extracted RNA was done using an Eppendorf BioPhotometer Plus spectrophotometer (Eppendorf, Hørsholm, Denmark). Samples were stored in RNase-free water at − 80 °C.

Real-time reverse transcription polymerase chain reaction (qPCR) was done by use of StepOne Software version 2.3 (Applied Biosystems, www.thermofisher.com). Reactions took place on 96-well optical plates using 5 mL Power SYBR® Green RT-PCR Mix (2×) (Applied Biosystems, www.thermofisher.com), 2 μL of cDNA (conc. 3 μg/mL) and primer concentrations of 0.1 μm each (final reaction volume was 10 μL). The gene-specific primers used in this experiment had previously been established and verified by other researchers (*see* Table 1 *of primers and their references*). All samples were run in duplicates with a non-template control on each plate. The reaction conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of PCR amplification was confirmed by melting curve analysis (95 °C for 15 s, 60 °C for 60 s and then 95 °C for 15 s). Rpl2, Ef1 α and RPS18 were evaluated as reference genes using the software DataAssist™ (Life Technologies 2012, version 3.0) whereof the Ef1 α was found to be the most stable.

Statistical analysis

The in vitro data was analysed by regression analyses using Statgraphics Centurion XVI software (16.0.07 version). Effect of TTA treatment was evaluated by one-way analyses of variance (ANOVA). Significant differences between means were evaluated by applying Duncan multiple range tests. If not significantly different, doses were pooled and analysed by one-way ANOVA or non-parametric tests of the medians. Relative gene expression of the in vivo study *II* and normalisation was done in regard to the reference gene Ef1 α using DataAssist™ software. A mixed effect model was then applied in *R* (version 2.15.0.) for evaluation

Table 1 Applied primers and their references

Short name	Genes	References
Nkx2.5	Homeobox protein Nkx-2.5	Grammes et al. 2012a, b; Castro et al. 2013
PCNA	Proliferating cell nuclear antigen	Castro et al. 2013
Srebp1	Sterol regulatory element binding protein 1	Schiller Vestergren et al. 2012
Srebp2	Sterol regulatory element binding protein 2	Schiller Vestergren et al. 2012
PGC1a	PPARγ cofactor 1a	Castro et al. 2013
AMPK	5-AMP-activated protein kinase	Castro et al. 2013
UCP2	Uncoupling protein 2	Zhou et al. 2012
D5	Δ5-desaturase	Schiller Vestergren et al. 2011
D6	Δ6-desaturase	Schiller Vestergren et al. 2011
Elovl2	Fatty acid elongase 2	Schiller Vestergren et al. 2011
Elovl5a	Fatty acid elongase 5	Schiller Vestergren et al. 2011
CD36	Cluster of differentiation 36	Schiller Vestergren et al. 2011
CPT1a	Carnitine palmitoyltransferase 1A	Schiller Vestergren et al. 2011
PPARα	Peroxisome proliferative activated receptor, alpha	Schiller Vestergren et al. 2011
$PPAR_{\beta}$	Peroxisome proliferative activated receptor, beta	Schiller Vestergren et al. 2011
PPARγ	Peroxisome proliferative activated receptor, gamma	Schiller Vestergren et al. 2011
ACO	Acyl-CoA oxidase	Schiller Vestergren et al. 2011
Ef1a	Eukaryotic translation elongation factor 1 alpha 1	Schiller Vestergren et al. 2011; Grammes et al. 2012a, b
Rpl2	RNA polymerase 2	Schiller Vestergren et al. 2012
RPS18	40S ribosomal protein S18	Castro et al. 2013

of the normalised ΔcT-values in regard of effect of treatment, sex and the interaction between these variables (*see* Dessen et al. 2016). In the in vivo *study I*, the GLM procedure with sampling date as the class variable within each treatment (TTA and control) followed by Duncan's multiple range test for differences between means was applied. Significance level was set to $P \leq 0.05$ for all analyses, and $P < 0.10$ was considered to be a trend. The proportion of the total variation explained by models is expressed by R^2 and calculated as the marginal contribution of the mean square of the parameter (type III sum of squares for ANOVA). Results are presented as the mean ± SEM (standard error of the mean) if not specifically stated otherwise.

Results

In vivo study I

In this study, TTA was administrated in the feed for the post-smolt during the first 10 weeks after sea transfer (15 April to 24 June). The CSI decreased significantly for both dietary groups during the first 6 weeks after sea transfer (15 April to 27 May). No reduction in CSI was observed among the TTA administrated fish from 27 May to 24 June, whereas a further significant decrease in CSI was detected in the control group during this period (Fig. 1a). The different time-dependant changes in heart index between the dietary groups coincided with the previously reported lower muscle fat content (TTA = 3.7 ± 0.1%, control = 4.5 ± 0.2%, $P = 0.01$) and lower CF (Fig. 1b) for the TTA group compared to the control group on the June 24th sampling (see Dessen et al. 2016). At this sampling point, the dietary administration of TTA ended and was in total equal to 0.2‰ of the initial biomass (*w*/*w*) of the TTA group. At the end of the experiment, 1 month later, the CSI of the control group increased and became similar to the TTA group. Significant effect of sex relating to mean CF within the TTA group (*see* Dessen et al. 2016) was corrected for by calculating the overall mean of the average male and female parameter.

In vitro study

Uptake and incorporation of 1-14C PA in heart cells in culture

In this experiment, salmon heart cells were pre-stimulated with increasing doses of TTA with the purpose of studying the effect of TTA on fatty acid uptake and β-oxidation in absence of endogenous or systemic factors. After incubation for 36 h with 1-^{14}C PA, total uptake of PA in cardiomyocytes was calculated as the sum of radioactivity found in cellular lipids and oxidation products (CO_2 + ASP nmol/mg protein). Regression analyses revealed a slight but significant positive linear relationship between the dose of TTA and total PA in cell lipid (total PA in cell lipid = 33.04 + 0.40 × TTA dose) and total uptake of PA (total PA uptake = 36.82 + 0.46 × TTA dose) measured as nanomoles per milligram protein (Tables 2 and 3). However, the one-way ANOVA test did not detect significant effects of the TTA dose, but a trend towards differences was observed (Tables 2 and 3). As the levels of lipid uptake and total cell lipid in doses 0 to 60 μM were not statistically different, they were pooled as one group and tested against the 120-μM dose. These analyses showed that the highest TTA dose had significant largest uptake of PA and incorporation of PA in the total cell lipid (Fig. 2).

The distribution of the incorporated 1-^{14}CPA in the analysed lipid classes was also found to be significantly affected by TTA dosage. Linear regression analyses revealed significant fit on the distribution of 1-^{14}CPA as percentage of total lipids in the two major lipid classes phospholipids and triacylglycerol, where PA was found in increasing amounts in PL and decreasing amounts in TAG with increasing dose of TTA (Fig. 3). However, the incorporation of 1-^{14}CPA in TAG was only significantly lower in the highest dose of TTA compared to the control and lowest dose of TTA (30 μM), but not so for TTA dose of 60 μM (Table 3). The highest level of 1-^{14}CPA in the free fatty acids was found in the 120-μM dose of TTA (Table 3). No effect of TTA dose was found in incorporation of PA in monoacyl- and diacylglycerides. However, when statistically pooling the TTA doses and testing these against the control, the incorporation of 1-^{14}CPA in monoglycerides was about 1.8 times higher in the pooled TTA group (one-way ANOVA $n = 3$ and 8: 1.8 vs 3.1%, $P = 0.04$, $R^2 = 0.31$). A similar test of diacylglycerides did not detect any difference.

Oxidation of 1-14C PA

No linear relationship to or statistical differences between treatments were found when CO_2 and acid-soluble products were calculated as percentages of total 1-^{14}C PA uptake (Table 2). As no significant difference was found among TTA treatments, these treatments were statistically pooled and tested as one TTA group vs the control (see above). Mean CO_2 derived from 1-^{14}C PA in the pooled TTA treatments was found to be about 1.6 times higher than the control (3.9 vs 6.4%, $P = 0.02$, $R^2 = 0.42$) which indicates a higher complete percentage oxidation of 1-^{14}C PA. ASP was not found to be significantly different when tested as pooled TTA treatments vs the control. However, when related to cell protein content (nmol/mg protein), heart cells pre-stimulated with 120 μM TTA had a significant higher release of 1-^{14}C CO_2 compared to the control and the lowest dose of TTA (Table 2). Similarly, formation of ASPs tested as nanomoles per milligram protein was significantly higher in the 120-μM dose compared to all the other treatments (Table 2). The regression analyses of the formation of 1-^{14}C PA-derived CO_2 and ASPs (in nmol/mg protein) were found to be significantly positive linearly correlated to the dosage of TTA ($CO_2 = 1.66 + 0.03 \times$ TTA dose, ASP = 2.12 + 0.03 × TTA dose) (Table 2).

Analysing total oxidation of 1-^{14}C PA as percentage related to total lipid uptake, no significant differences were found among treatments (Table 2). Thus, the TTA treatments were statistically pooled and tested vs the control. A one-way ANOVA test did not detect any difference, but a non-parametric test (Mann-Whitney/Wilcoxon *W* test) showed that the median of the control group was significantly lower compared to the median of the pooled TTA group (7.9 vs 11.7, $W = 24.0$, $P = 0.019$). When related to cell protein content (nmol/mg protein), total oxidation was positively linearly related to TTA dosage (total oxidation = 3.77 + 0.06 × TTA dose) in a dose-dependent manner and total oxidation was highest in the 120-μM dose vs all the other treatments (Table 2).

To describe β-oxidation in the two cell compartments mitochondria and peroxisomes, the ASP were partitioned into fractions by HPLC (Table 2): oxaloacetate/malate, acetate, aceto-acetate, β-hydroxybutyrate and β-hydroxy-β-methylglutaric acid. No relation to dose or differences was found in these parameters between the treatments. Statistical tests of pooled groups

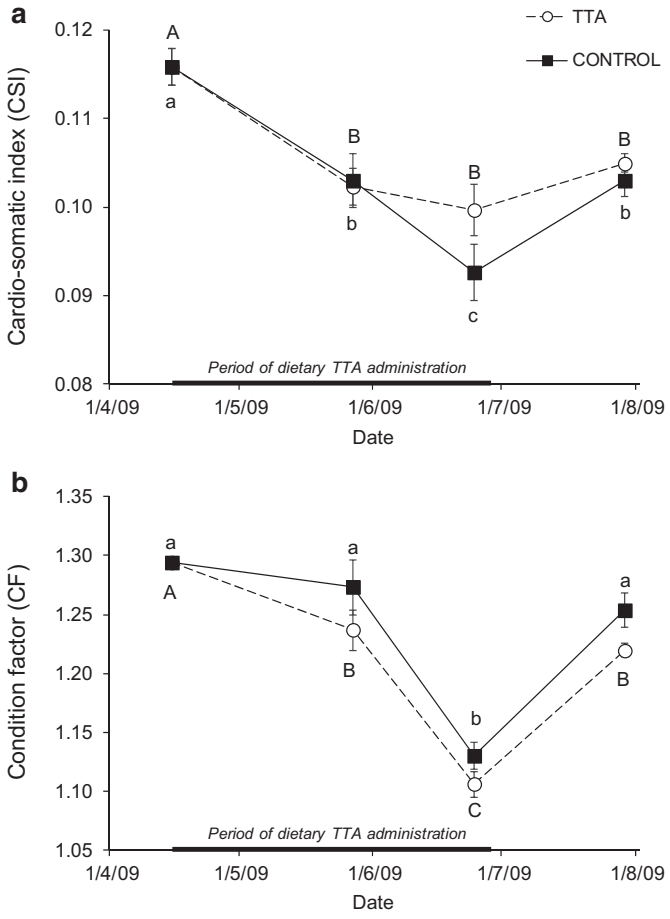

Fig. 1 Changes in mean ± S.E ($n = 3$) cardio-somatic index (**a**) and condition factor (**b**) of Atlantic salmon post-smolt given a diet supplemented with tetradecylthioacetic acid (TTA) or a non-supplemented control diet (control) during 15 weeks after sea transfer (15 April to 29 July). Different upper case letters indicate significant differences ($P < 0.05$) between sampling points within the TTA group. Different lower case letters indicate significant differences ($P < 0.05$) between sampling points within the control group. The period of dietary TTA administration (15 April to 24 June) is indicated by the bold line at the timeline axis (x-axis)

(doses 0–60 vs 120 μM or control vs pooled TTA treatments) did not detect any pooled group differences.

Recovery was calculated as the sum recovered of the added radioactivity in total lipids, CO_2 and ASP per milligram protein. There was a tendency of higher recovery in the highest dose of TTA ($P = 0.10$). The overall mean recovery of 1-^{14}C PA in the heart cells was 32.8 ± 5.5% (SEM) of the added radioactivity to the medium.

In vivo study II

The purpose of the in vivo (II) experiment was to further evaluate possible treatment effects on genes involved in fatty acid metabolism and cell growth in the salmon heart. But statistical evaluation of fish receiving the 115-μg/kg TTA dose showed that the results in this group deviated from the other treatments in such a way that it was decided to omit the results in this group from this study. One possible explanation may be inadequate injections, as the fish in this group were not seen to behave differently than fish in the other groups. Mortality in this experiment was one fish only receiving the 231-μg/kg TTA dose.

When applying the full statistical model on relative mRNA levels, only marginal or no significant differences between the sexes or interaction between treatment and sex were found (results not shown). The model was therefore reduced to only include the treatment variable.

The investigated genes directly involved in fatty acid ß-oxidation showed a diverse picture: Acyl-CoA

Table 2 Uptake of 1-14C palmitic acid, oxidation and oxidation products (acid-soluble products, ASP) in salmon heart cells in culture (mean ± pooled SEM, $n = 11$) and the probability (P value) and the total variation explained (R^2) by the model used in the statistical analyses (linear regression and ANOVA). Different superscript letters indicate significant differences ($P < 0.05$) across rows

Sample		Control	TTA30	TTA60	TTA120	Pooled SEM	Regression		ANOVA	
							P value	R^2	*P* value	R^2
Total uptake	(nmol/mg protein)	49.42	40.79	48.69	99.67	± 10.1	*< 0.03*	0.38	0.10	0.39
CO_2	(% of uptake)	3.89	6.78	6.49	5.87	± 0.5	0.34	0.07	0.14	0.32
ASP	(% of uptake)	4.08	9.33	6.64	5.54	± 0.9	0.99	< 0.01	0.17	0.27
Oxidated	(% of uptake)	7.96	16.10	13.13	11.41	± 1.4	0.74	< 0.01	0.17	0.27
Total CO_2	(nmol/mg protein)	1.94^{b}	2.52^{b}	3.04^{ab}	5.81^{a}	± 0.6	*< 0.01*	0.61	*0.04*	0.54
Total ASP	(nmol/mg protein)	2.00^{b}	3.35^{b}	3.02^{b}	5.37^{a}	± 0.5	*< 0.01*	0.60	*0.03*	0.57
Oxidated	(nmol/mg protein)	3.94^{b}	5.87^{b}	6.06^{b}	11.18^{a}	± 1.1	*< 0.01*	0.62	*0.03*	0.56
ASP fractions										
Oxalacetate/malate	(% of total ASP)	89.11	82.46	88.00	90.04	± 2.0	0.58	< 0.01	0.56	< 0.01
Acetate	(% of total ASP)	6.42	9.18	7.37	7.37	± 0.7	0.94	< 0.01	0.58	< 0.01
Aceto-acetate	(% of total ASP)	1.80	3.70	1.83	0.24	± 1.0	0.42	< 0.01	0.73	< 0.01
β-hydroxybutyrate	(% of total ASP)	1.40	2.77	1.22	1.01	± 0.7	0.63	< 0.01	0.82	< 0.01
Beta-hydroxy-beta-methylglutarate	(% of total ASP)	1.26	1.88	1.58	1.34	± 0.5	0.97	< 0.01	0.98	< 0.01

Total uptake was calculated as the radioactivity in cellular lipids + CO_2 + ASP

Table 3 Incorporation of 1-14C palmitic acid in total cell lipid, distribution in analysed lipid classes in salmon heart cells in culture (mean ± pooled SEM, $n = 11$) and the probability (P value) and the total variation explained (R^2) by the model used in the statistical analyses (linear regression and ANOVA). Different superscript letters indicate significant differences ($P < 0.05$) across rows

Sample		Control	TTA30	TTA60	TTA120	Pooled SEM	Regression		ANOVA	
							P value	R^2	*P* value	R^2
Total cell lipid	(nmol/mg protein)	45.48	34.92	42.62	88.49	± 9.1	*0.03*	0.35	0.11	0.37
Phospholipids	(% of total lipid)	26.36^{ab}	18.91^{b}	27.7^{ab}	35.57^{a}	± 2.4	*0.04*	0.32	*0.04*	0.53
Triacylglycerides	(% of total lipid)	21.71^{ab}	22.95^{a}	17.4^{bc}	17.14^{c}	± 1.0	*0.02*	0.39	*0.05*	0.51
Diacylglycerides	(% of total lipid)	0.76	0.60	0.57	0.51	± 0.1	0.31	0.01	0.67	< 0.01
Monoacylglycerides	(% of total lipid)	1.77	2.72	3.85	3.21	± 0.3	0.11	0.18	0.14	0.31
Free fatty acids	(% of total lipid)	2.46^{b}	2.23^{b}	2.44^{b}	3.19^{a}	± 0.1	*0.01*	0.44	*0.04*	0.54

oxidase (ACO), which is regarded to be regulating the peroxysomal ß-oxidation, was significantly more upregulated, whereas the carnitine palmitoyltransferase 1 (CTP1), which regulates fatty acid transport into the mitochondria, was not influenced when the mRNA levels where compared to the control (Fig. 4a). mRNA level generated by genes coding for fatty acid desaturase and elongation (Δ5-desaturase, Δ6-desaturase and Elovl2, Elovl5) and sterol-binding proteins (SREBP1,2) were higher in treated fish (Fig. 4b). The same was observed in the two genes involved in cell growth and proliferation: NKX2.5 and PCNA which both were significantly upregulated at all TTA doses as well as the PGC1 which is involved in DNA replication was upregulated, but only significantly at the lowest dose (Fig. 4c).

The well-known regulator family of fatty acid β-oxidation and energy homeostasis, the peroxisome proliferative-activated receptors (PPARs), seemed to have been affected differently by the treatments: The PPARα was not upregulated in the treated fish hearts whereas the $PPAR_{\beta}$ was clearly more upregulated (Fig. 4d). PPARγ was not found to respond to the TTA treatment. Regarding uptake and transport of fatty acids across the cell membrane (CD36 and UCP2), the mRNA level in hearts of treated fish was generally lower compared to the control—however, not statistically different (Fig. 4a).

The 5-AMP-activated protein kinase (AMPK) was upregulated in the TTA-treated fish (Fig. 4d). In the investigations of relationships between increasing doses of TTA and effect on gene expression, regression analyses on the relative ΔcT data only revealed weak correlation between dose of TTA and respective level of mRNA (results not shown).

Discussion

The first study in vivo (*I*) demonstrated that dietary treatment with TTA in a period after transfer to seawater enhances the ability of salmon post-smolts to maintain a significantly higher CSI, as compared to controls. In rat studies, TTA has been shown to result in proliferation of liver mitochondria and peroxisomes and increased liver size (Berge et al. 1989). Similarly, in salmon given TTA-supplemented diets, increased liver size has been

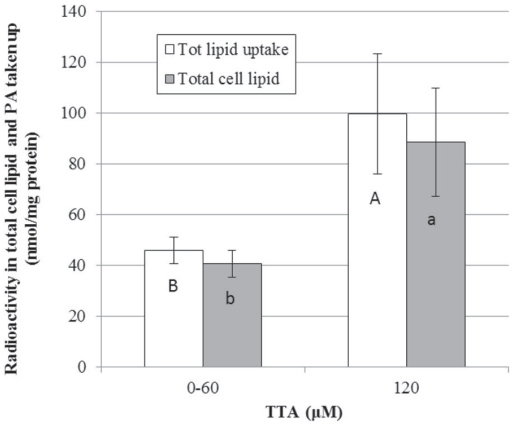

Fig. 2 Total 1-14C palmitic acid (PA) uptake (nmol/mg protein) and total 1-14C PA (nmol/mg protein) in cell lipid in heart cells in culture, pre-incubated with increasing doses of TTA (μM). Statistically pooled doses from 0 to 60 μM ($n = 8$) were tested against dose 120 μM ($n = 3$). Different upper case letters indicate significant differences ($P = 0.01$, $R^2 = 0.51$) in total 1-14C PA uptake. Different lower case letters indicate significant differences ($P = 0.01$, $R^2 = 0.49$) in 1-14C PA in total cell lipid. Error bars are standard error of the means (SEM)

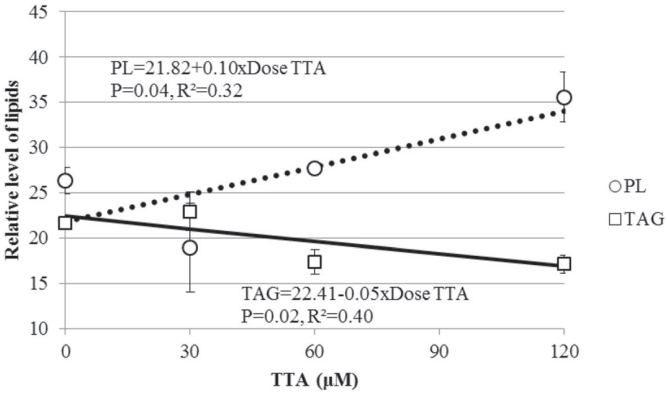

Fig. 3 Linear regression on the distribution of 1-14C palmitic acid (PA) in phospholipids (PL) (dotted line) and triglycerides (TAG) (solid line) relative to the total cell 1-14C PA in heart cells cultivated for 36 h. The heart cells were pre-stimulated by incubation for 3 days with increasing doses of TTA (μM) in the culture medium before addition of 1-14C PA. The indicated values are mean values ($n = 3$). Error bars are the standard error of the mean (SEM)

documented (Kleveland et al. 2006). A similar induced proliferation of mitochondria and/or peroxisomes is most probably the explanation for the larger heart after TTA feeding found in the present study. TTA has previously been shown to result in higher fatty acid oxidation in mammals (Berge et al. 1989, 2002; Hvattum et al. 1993) and recently also in salmon (Moya-Falcon et al. 2004; Alne et al. 2009; Grammes et al. 2012a, b; Dessen et al. 2016). The lower condition factor seen in the TTA-treated fish also confers with stimulated expenditure of energy reserves in salmon during this period.

Cardiac metabolism in salmon has been sparsely investigated. Consequently, the possibility of studying short-term effects of TTA on salmon heart by pre-treatments of cardiomyocytes in culture was interesting. After 3 days of TTA stimulation, positive effects on

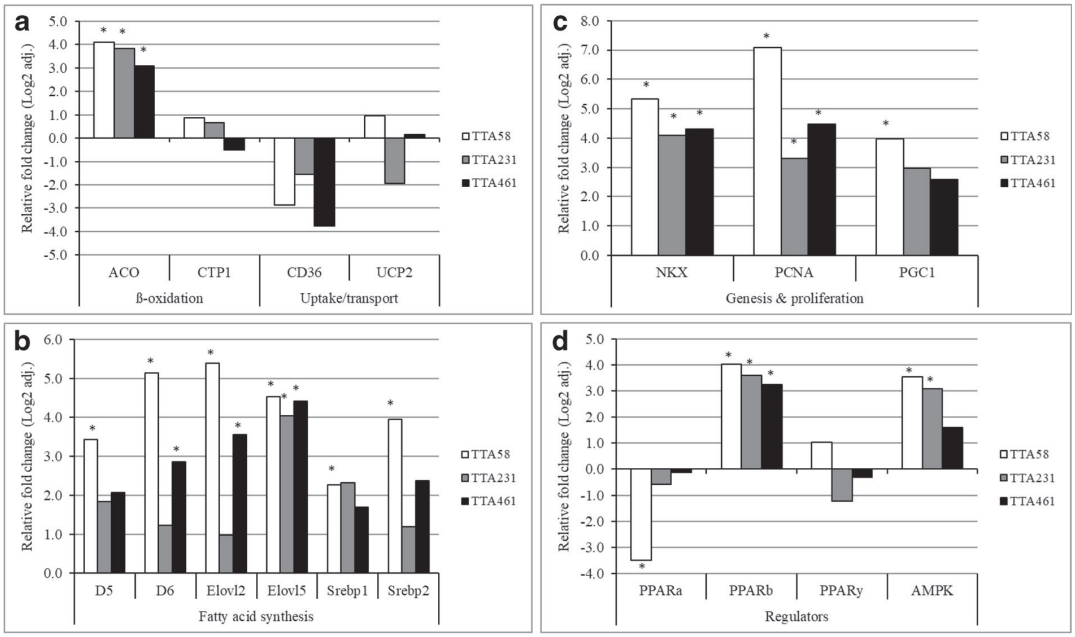

Fig. 4 **a**, **b**, **c** and **d** Relative mRNA levels (log 2 adjusted $2^{\wedge-\Delta\Delta cT}$ values) in heart ventricles of young Atlantic salmon 8 days past treatment with injections with increasing doses of TTA (58, 231 and 461 μg/kg). *Significant P value ($P < 0.05$) present in comparison to reference level in the control group which was subjected to injections with physiological saline only

palmitic acid uptake (Fig. 2) and oxidation to both CO_2 and ASP (Table 2) were seen at the highest dose used. Higher incorporation into cell lipids were further observed, but the relative amounts of PA oxidised or stored as lipids did not change as compared to the controls. More of the stored radioactivity was, however, recovered in the PL fraction and less in TG with increasing doses of TTA. This may be taken as an indication of organ proliferation, and such a suggestion may further be supported by the gene expression results in the in vivo (II) experiment. In the in vitro experiment, it was evident that the 120-μM dose had a large influence on the statistical evaluation of the data. Inclusion of other doses of TTA in future cell culture experiments may provide for a better understanding regarding the biological effects of this or similar compounds and perhaps more robust data especially for dose-response analyses that may be obtained.

Our attempts to distinguish between effects on mitochondrial and/or peroxisomal beta-oxidation by analysing the production of different acid-soluble products gave no clear answer. In the study with in vivo injection of TTA, a clear stimulation of ACO transcription was, however, recognised, while any effect on the mitochondrial CPT 1 transcription was not seen. This may suggest that at least the short-time effect of TTA on fatty acid oxidation in salmon hearts mainly is due to an increase in peroxisomes and peroxisomal ß-oxidation capacity. On the other hand, the gene PGC1a was clearly upregulated in this study and perhaps indicating a stimulation of mitochondrial biogenesis and increased beta-oxidation in this cell compartment (Jäger et al. 2007).

The peroxisome proliferator-activated receptors, the PPARs, have in studies with salmon been shown to be upregulated by TTA. Especially the expression of PPARα was shown to increase in salmon hearts after treated with TTA-feed for 8 weeks in sea (Grammes et al. 2012a) and a slight, but statistically not significant increase in $PPAR_{\beta}$ was further observed. In our short-time study, the expression of neither PPARα nor γ was enhanced by injection of TTA, PPARα even negatively affected at the lowest dose. Conversely, TTA significantly increased the amount of $PPAR_{\beta}$ mRNA by all three doses. $PPAR_{\beta}$ is known to stimulate fatty acid oxidation in rat cardiomyocytes (Gilde et al. 2003). In addition, $PPAR_{\beta}$ has also been found to be related to physiological cardiac hypertrophy (Grammes et al. 2012b) which may explain the increase of CSI observed in the in vivo *I* experiment. The activation of transcription factors like the NKX 2.5, PCNA and partly PGC1 seen in the injection study may also be taken to corroborate with this view.

Relative activity in PUFA synthesis seen as increased relative amount of mRNA derived from the elongation and desaturation genes Δ5, Δ6, Elovl2 and 5 as well as the sterol-binding proteins SREBP1 and 2, seemed higher in TTA-treated fish hearts. In rat hearts, a two-fold increase in 22:6 (n-3) and major decrease in 20:4 (n-6) have been found (Skrede et al. 1997). Similarly, Moya-Falcón et al. (2006) reported an accumulation of 22:6 (n-3) in cell membranes of salmon liver after TTA treatment. In the latter study, the authors related the accumulation to an increase in oxidation of other more utilisable fatty acids and thus a conservation of 22:6 (n-3) rather than an increase in desaturation and elongation of shorter chain n-3 fatty acids. Altogether, these effects may, in addition to the higher capacity of energy utilisation, indicate that hearts in TTA-treated fish are more robust and able to secure the need for healthy fatty acids.

Additionally, the investigated genes related to cell genesis/differentiation in this experiment were upregulated. The relative mRNA amount of 5-AMP-activated protein kinase seemed to be higher in TTA-treated fish, which may indicate lower energy status within the cell as compared to untreated fish. As noted above, lipid and protein synthesis seemed upregulated in the experiment in vivo; thus, a higher amount of AMPK may seem contradicting as the AMPK is believed to inhibit lipid synthesis when energy status within the cell is low (Castro et al. 2011; Polakof et al. 2011). On the other hand, AMPK may induce transcription or activate genes that are involved in protein synthesis (Hardie 2004) which perhaps can be interpreted as the role of AMPK in this experiment.

In conclusion, the three experiments seem to indicate a higher catabolic activity of fatty acids in the heart as a response to TTA. Such increase in cardiac efficiency may perhaps offer significant benefits for farmed Atlantic salmon, especially in energy-demanding situations such as after transfer from freshwater to seawater as in the in vivo *I* experiment. This may also be related to the significantly higher survival previously observed in TTA-treated S0 post-smolts during a natural outbreak of heart and skeletal muscle inflammation (Alne et al. 2009).

Compliance with ethical standards

Ethical concern The in vivo *study I* and the in vitro study were done in Norway and conducted according to the regulations for fish welfare set by the Norwegian Experimental Animal Authority. In the Faroe Islands, however, there is no legislation concerning experiments with animals, so the local "animal protection act" was adhered to throughout the in vivo *II study* (Vinnumálaráðið 1990). A fish veterinarian advised on best practice in relation to anaesthetization and injection procedures to ensure no undue suffering of the fish. There was no fish mortality caused by experimental procedures or management practice as effort was put into providing optimal welfare of the fish.

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