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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 separated by ' - '

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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 the sampling point 24 June~~, 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 $(g)^{-1} \times 100$, 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 ~~[1-14C]~~ 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, Ef1α and RPS18 were evaluated as reference genes using the software DataAssist™ (Life Technologies 2012, version 3.0) whereof the Ef1α 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 Ef1α using DataAssist™ software. A mixed effect model was then applied in *R* (version 2.15.0.) for evaluation

Q3 Q1 **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

Q4 Q5

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.

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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 1-14C 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 1-14CPA 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 1-14CPA 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 1-14CPA 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 1-14CPA 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 1-14CPA 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 1-14C 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 1-14C 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 1-14C 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 1-14C 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 1-14C PA-derived CO_2 and ASPs (in nmol/mg protein) were found to be significantly positive linearly correlated to the dosage of TTA ($CO_2 = 1.66 + 0.03$ × TTA dose, ASP = 2.12 + 0.03 × TTA dose) (Table 2).

Analysing total oxidation of 1-14C 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

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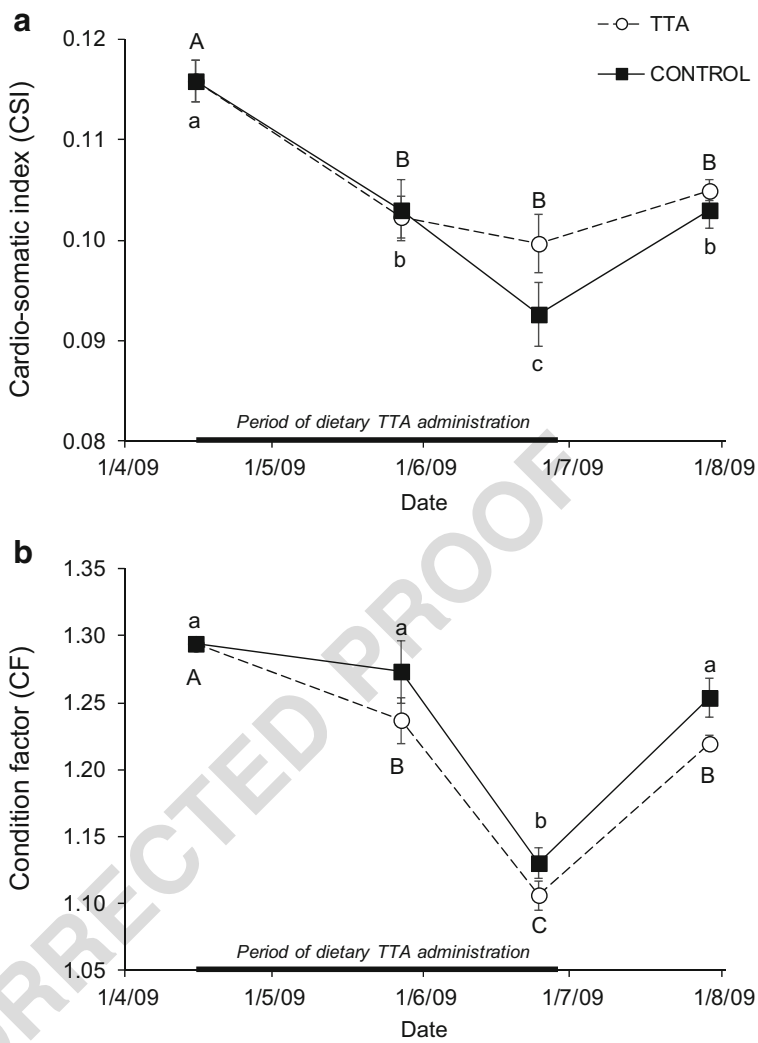

Fig. 1 Changes in mean ± S.E ($n = 3$) cardio-somatic index (**a**) and condition factor (**b**) of Atlantic salmon post-smolt given a diet supplemented with tetradecylthioacetic acid (TTA) or a non-supplemented control diet (control) during 15 weeks after sea transfer (15 April to 29 July). Different 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 ~~1-14C~~ 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

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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

t3.1 **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

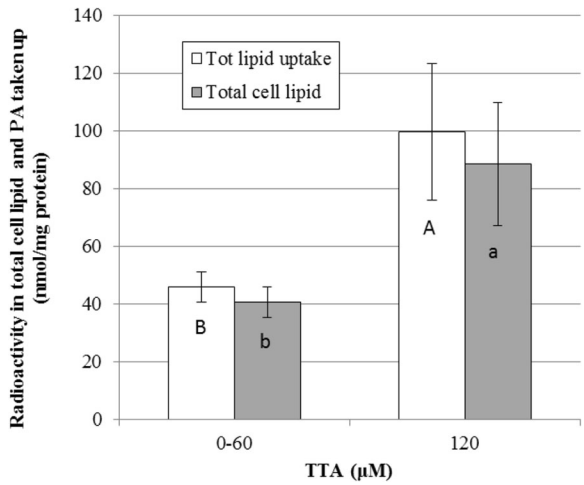

Fig. 2 Total 1-14C palmitic acid (PA) uptake (nmol/mg protein) and total 1-14C PA (nmol/mg protein) in cell lipid in heart cells in culture, pre-incubated with increasing doses of TTA (µM). Statistically pooled doses from 0 to 60 µM ($n = 8$) were tested against dose 120 µM ($n = 3$). Different upper 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)

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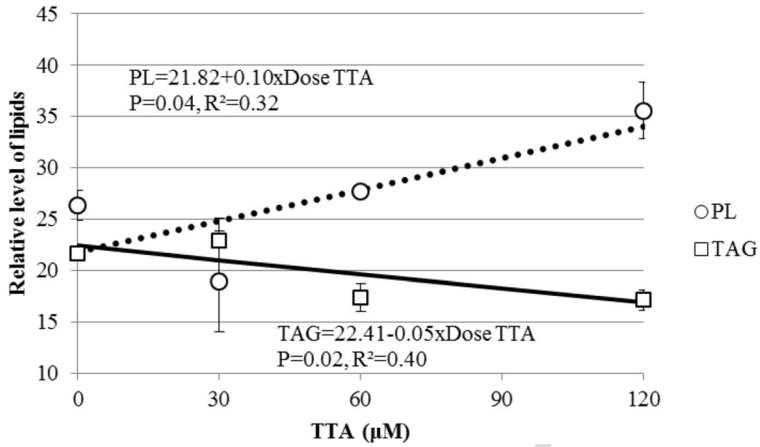

Fig. 3 Linear regression on the distribution of 1-14C palmitic acid (PA) in phospholipids (PL) (dotted line) and triglycerides (TAG) (solid line) relative to the total cell 1-14C PA in heart cells cultivated for 36 h. The heart cells were pre-stimulated by incubation for 3 days with increasing doses 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

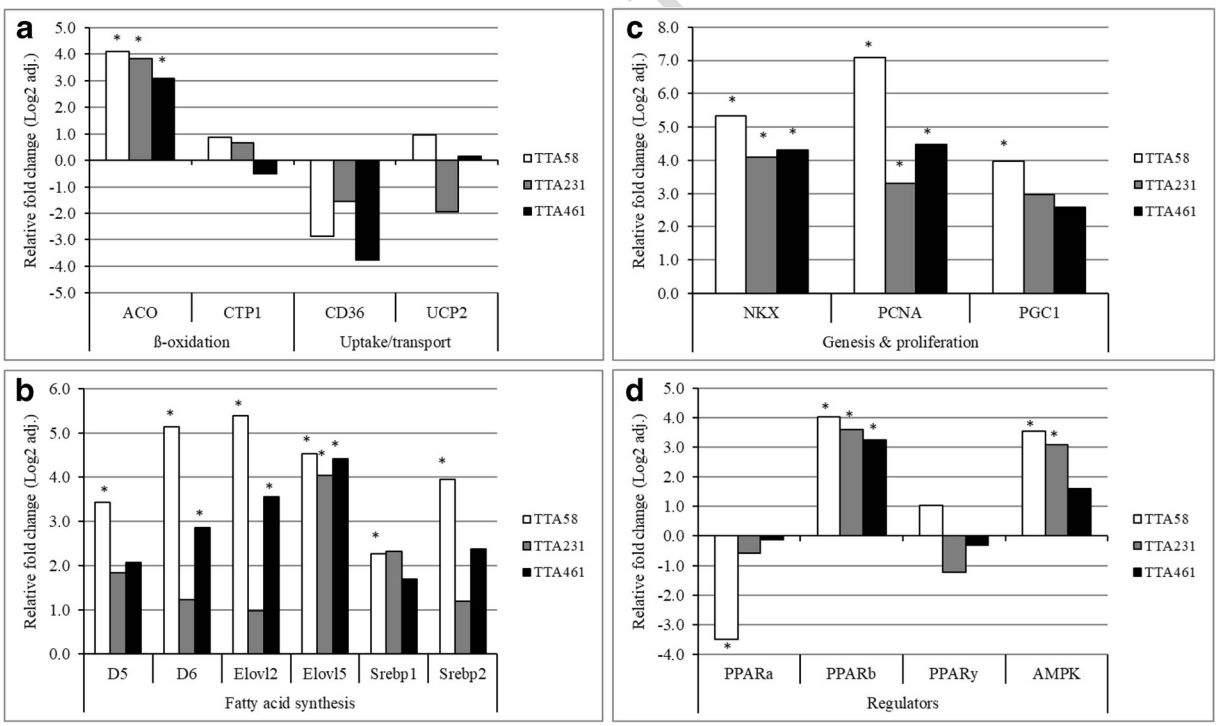

Fig. 4 **a**, **b**, **c** and **d** Relative mRNA levels (log 2 adjusted $2^{-\Delta\Delta cT}$ values) in heart ventricles of young Atlantic salmon 8 days past treatment with injections with increasing doses of TTA (58, 231 and 461 μg/kg). *Significant P value ($P < 0.05$) present in comparison to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

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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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AUTHOR QUERIES

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Q1. Please check if the affiliations are presented correctly.
Q2. There is an inconsistency with the usage of the term "1-14C". Please check if it is presented correctly.
Q3. Please check if the changes made in the tables are appropriate.
Q4. The citation “Grammes et al. 2012” has been changed to “Grammes et al. 2012a, b” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.
Q5. The citation “Zhou et al. 2011” has been changed to “Zhou et al. 2012” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.
Q6. Entries in bold were changed to italics as per journal instructions for Tables 2 and 3. Please check and provide the significance of their emphasis in the form of a table note.
Q7. The term "5’ AMP-activated protein kinase" was changed to "5-AMP-activated protein kinase". Please check if appropriate and intended meaning is retained.
Q8. Please provide complete bibliographic details of this reference: VKM (2014).