Influence of Dietary Oil Content and Conjugated Linoleic Acid (CLA) on Lipid Metabolism Enzyme Activities and Gene Expression in Tissues of Atlantic Salmon (*Salmo salar* L.)

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Abbreviations: CPT-I, carnitine palmitoyl transferase-I; PPAR, peroxisome proliferator-activated receptor; SCD, stearoyl CoA desaturase; SREBP, steryl regulatory element binding protein.

ABSTRACT: The overall objective is to test the hypothesis that conjugated linoleic acid (CLA) has beneficial effects in Atlantic salmon through affecting lipid and fatty acid metabolism. The specific aims of the present study were to determine the effects of CLA on some key pathways of fatty acid metabolism including fatty acid oxidation and highly unsaturated fatty acid (HUFA) synthesis. Salmon smolts were fed diets containing two levels of fish oil (low, ~18% and high, ~34%) containing three levels of CLA (a 1:1 mixture of 9-cis,trans-11 and trans-10,cis-12 at 0, 1 and 2% of diet) for 3 months. The effects of dietary CLA on HUFA synthesis and β-oxidation were measured and the expression of key genes in the fatty acid oxidation and HUFA synthesis pathways, and potentially important transcription factors, peroxisome proliferators activated receptors (PPARs), determined in selected tissues. Liver HUFA synthesis and desaturase gene expression was increased by dietary CLA and decreased by high dietary oil content. Carnitine palmitoyltransferase-I (CPT-I) activity and gene expression were generally increased by CLA in muscle tissues although dietary oil content had relatively little effect. In general CPT-I activity or gene expression was not correlated with β-oxidation. Dietary CLA tended to increase PPARα and β gene expression in both liver and muscle tissues, and PPARγ in liver. In summary, gene expression and activity of the fatty acid pathways were altered in response to dietary CLA and/or oil content, with data suggesting that PPARs are also regulated in response to CLA. Correlations were observed between dietary CLA, liver HUFA synthesis and desaturase gene expression, and liver PPARα expression, and also between dietary CLA, CPT-I expression and activity, and PPARα expression in muscle tissues. In conclusion, this study suggests that dietary CLA has effects on fatty acid metabolism in Atlantic salmon and on PPAR transcription factors. However, further work is required to assess the potential of CLA as a dietary supplement, and the role of PPARs in the regulation of lipid metabolism in fish.

CLA is a term used to describe conjugated isomers of linoleic acid (18:2n-6), the two main naturally occurring isomers being cis-9,trans-11 and trans-10,cis-12. In mammals, dietary CLA has been shown to have a number of physiological effects and health benefits including anticarcinogenic and immune enhancing properties (1). In addition, CLA has several beneficial effects on lipid metabolism in mammals, including altering body composition (2). Specifically, CLA decreased body fat and increased lean body mass in mice, rats and pigs (2). Decreased body fat has also been observed in human studies although the effect was much less than that observed with mice (3). CLA is also known to decrease the activity and gene expression of mammalian stearoyl CoA Δ9 desaturase (4) and may also suppress Δ6 and Δ5 desaturase (5) and elongase (6).

There is accumulating evidence that some of the effects of CLA may be mediated through transcription factors such as peroxisome proliferator activated receptors (PPARs) or sterol regulatory element binding proteins (SREBPs). Specifically CLA isomers are ligands and activators of both PPARα (7) and PPARγ (8) that in mammals are, in turn, known to regulate the expression of genes of fatty acid oxidation and lipid deposition in liver and adipose tissue, respectively (9). Dietary CLA also influenced the expression of SREBPs in ob/ob mice (10). Both PPARs and SREBPs are involved in the control of fatty acid desaturation and elongation, with PPARα and SREBP-1 both affecting Δ6 and Δ5 activities (11), and SREBP-1 also regulating elongase activities (12). Therefore, CLA via PPARs and/or SREBPs may affect lipid contents and fatty acid compositions in a variety of ways including selectivity in oxidation and deposition of specific fatty acids and modulation of fatty acid desaturation and elongation.

The overall objective is to test the hypothesis that CLA has beneficial effects in Atlantic salmon through affecting lipid and fatty acid metabolism. The specific aims of the present study were to determine the effects of CLA on some key pathways of fatty acid metabolism including fatty acid oxidation and highly unsaturated fatty acid (HUFA) synthesis via desaturation and elongation. To this

end, salmon smolts were fed diets containing two levels of fish oil (low, ~18% and high, ~34%) containing three levels of CLA (a 1:1 mixture of the two main isomers present in nature, 9-cis,trans-11 and trans-10,cis-12. at 0, 1 and 2% of diet) for 3 months. The effects of dietary CLA on the expression of key genes of fatty acid oxidation and HUFA synthesis, and on the enzymic activities of the pathways were determined. In addition, the effect of CLA on the expression of potentially important transcription factors, PPARs, was determined in selected tissues.

MATERIALS AND METHODS

Diets and animals. Atlantic salmon smolts ($S^1/_2$) were obtained from a commercial salmonid farm (Howietoun Fish Farm, Sauchieburn, Scotland) in late October and transported to Stirling University, Institute of Aquaculture, Marine Environmental Research Laboratory, Machrihanish, Scotland. The fish were maintained in stock tanks for three weeks at ambient water temperature of around 10-11 ^oC to acclimatize during which time the fish were fed standard salmon diet, before being randomly distributed between eighteen indoor, round, conical tanks of 1.5m^3 volume (1.72m diameter). The initial stocking density was 100 fish of average fish weight 87.5 ± 1.6 g per tank (5.8 kg/m^3). Water temperature was maintained at 12 ^oC (±1 ^oC) throughout the trial, with a light regime of 12L:12D. Six experimental diets were fed to triplicate tanks for 3 months, with feed supplied to appetite manually. The experimental diets were formulated to satisfy the nutritional requirements of salmonid fish (13), and were formulated and manufactured by BioMar A/S, Brande, Denmark. The trial had a 3 x 2 factorial design with CLA added at three concentrations to diets with two oil contents (low and high). Thus diets were produced with 0, 1 and 2% CLA replacing standard Northern hemisphere fish oil in smolt feeds containing either 18 or 34% total lipid. Diets were formulated to be isonitrogenous and so protein content was constant between diets of different oil content (Table 1). Diets within the low or high oil groups were identical in formulation other than fatty acid composition with CLA (LUTA-

CLA^{TM} 60, containing 60% CLA methyl esters as a 50:50 mixture of c9, t11 and t10, c12 isomers; BASF AG, Ludwigshafen, Germany) balanced by fish oil (capelin oil, Norsemeal Ltd., London, UK). The fatty acid compositions of the diets are presented in Table 2.

Sampling protocols. At the termination of the trial, ten fish per dietary treatment (3-4/tank) were individually weighed, eviscerated and hepato- and viscero-somatic indeces determined. Samples of 0.5g of liver, white and red muscle were rapidly dissected from six of the fish (two per tank) and immediately frozen in liquid nitrogen for RNA analyses. In addition, samples of 1-2g of liver, white and red muscle for biochemical analyses were collected from the same fish and immediately frozen in liquid nitrogen. All samples were subsequently stored at –80 ^{o}C prior to analyses.

RNA extraction and real-time quantitative-polymerase chain reaction (rtqPCR). Total RNA was isolated from 100 - 500 μg (depending upon tissue) of pooled (2 fish) tissue by the standard TRIzol method (Invitrogen Ltd, Paisley, UK), and DNA removed using DNA-*free*™ DNase treatment (Ambion Inc., Austin, TX, USA). Five μg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega UK, Southampton, UK). The PCR primers were designed according to the salmon cDNA sequences for Δ6 desaturase (accession no. AY458652), Δ5 desaturase (accession no: AF478472), CPT-I, PPARα (M.J. Leaver, personal communication), PPARβ (accession no: AJ416953) and PPARγ (accession no: AJ416951). Primer sequences and PCR product sizes are given in Table 3. The linearised plasmid DNA containing the target sequence for each gene was quantified to generate a standard curve of known copy number. Amplification of cDNA samples and DNA standards was carried out using the SYBR Green PCR Kit (Applied Biosystems, Foster City, California, USA) with the following conditions: denaturation for 15 min at 95 ^{o}C followed by 45 cycles of 15 s at 95 ^{o}C, 15 s at 56 ^{o}C and 30 s at 72 ^{o}C, followed by product melting to check purity of PCR product. Thermal cycling and fluorescence detection were conducted using the Rotor-Gene 3000 system (Corbett Research, Cambridge, UK). Expression of

target genes was normalised using Quant-iT^{TM} High Sensitivity DNA Assay kit containing PicoGreen reagent, (Molecular Probes, Rijinsburgerweg, The Netherlands) using a modified version of the manufacturers protocol (14). All reactions were carried out in duplicate and a linear standard curve was drawn, plotting the average absorbance values obtained for the Quant-iT^{TM} DNA values against the standard DNA concentration, with the concentration of total cDNA calculated from the linear equation.

Fatty acid desaturation and elongation (HUFA synthesis) in liver microsomes. Portions of frozen liver were homogenised to 10% (w/v) in 0.25 M sucrose in 40 mM phosphate buffer pH 7.4 containing 1 mM EDTA, 0.15 M KCl, 40 mM KF and 1 mM N-acetyl cysteine. The homogenate was centrifuged at 25 000 g for 15 min at 4 ºC, the floating fat layer removed by aspiration and the remaining supernatant centrifuged at 105 000 g for 60 min at 4 ºC. The floating fat layer was aspirated and the supernatant decanted. The pelleted microsomal fraction was resuspended in 1 ml of sucrose buffer and 50 µl taken for protein determination according to Lowry et al. (15) after incubation with 0.45 ml of 0.25% (w/v) SDS/1M NaOH for 45 min at 60ºC. The assay mixture, in sucrose buffer pH 7.4, contained 5 mM $MgCl_2$, 1.5 mM glutathione, 0.5 mM nicotinamide, 1 mM NADH, 100 µM coenzyme A, and 5 mM ATP in a total volume of 0.75 ml. Fifty µl of [1-^{14}C]18:3n-3 (0.25 µCi, 5 µM final concentration), as a fatty acid-free (FAF) BSA complex, was added and the reaction initiated by the addition of 200 µl of microsomes. Incubation continued for 1 h at 20 ºC and the reaction was stopped by the addition of 5 ml of chloroform/methanol (2:1, v/v) containing 0.01% BHT, and total lipid extracted, transmethylated and FAME prepared as described previously (16). FAME were separated by argentation TLC, visualized by autoradiography and radioactivity determined by scintillation counting as described previously (17).

Assay of carnitine palmitoyltransferase I (CPT-I) and fatty acyl β-oxidation in tissue homogenates. Liver, red and white muscle were weighed, diced and homogenised to 20% (w/v) in 0.25 M sucrose in 10 mM N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES) buffer and 1 mM EDTA, pH

7.4. The homogenates were centrifuged at 1880 x g for 10 min at 4˚C, the floating fat layer aspirated and the post-nuclear fractions collected, 50 μl taken for protein determination as above, and portions used immediately for determination of enzyme activities. CPT-I activity was estimated by determining the production of palmitoyl[^{3}H]carnitine from palmitoyl CoA and [^{3}H]carnitine essentially as described by Saggerson and Carpenter (18). Fatty acyl β-oxidation was estimated by determining radioactivity in acid soluble products derived from the oxidation of [1-^{14}C]palmitoyl-CoA as described by Torstensen et al. (19).

Materials. [1-^{14}C] Palmitoyl CoA (50-55 mCi/mmol) and [methyl-^{3}H] L-carnitine hydrochloride (60-86 Ci/mmol) were obtained from GE Healthcare Bio-Sciences (Little Chalfont, Bucks, U.K.), and [1-^{14}C]18:3n-3 (50-55 mCi/mmol) was obtained from NEN (Perkin Elmer LAS (UK) Ltd., Beaconsfield, U.K.). BHT, carnitine, FAF-BSA, HEPES, palmitoyl-CoA, silver nitrate and TriReagent were obtained from Sigma Chemical Co. (Poole, U.K.). TLC (20 cm x 20 cm x 0.25 mm) plates, precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Fisher Scientific UK, Loughborough, England.

Statistical analysis. All data are presented as means ± SD (n value as stated). Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. The effects of dietary CLA and oil content were determined by two-way ANOVA with Bonferroni post-tests to determine significance of differences due to CLA. Differences were regarded as significant when $P < 0.05$ (20).

RESULTS

Diet composition, growth and biometry. Inclusion of CLA in the low oil diets resulted in levels of total CLA of 5.9% and 9.5% of total fatty acids at the 1 and 2% inclusion levels, respectively (Table 2).

Obviously, in the high oil diets, inclusion of CLA at 1 and 2% resulted in lower levels of total CLA, at 3.3% and 5.8% of total fatty acids, respectively. The levels of CLA in relative terms were identical in the L1 and H2 diets with an overall rank order for CLA content of L2 > L1 = H2 > H1 > L0/H0. There were no effects of diet on growth parameters, with no significant effects of CLA or oil content on final weights or specific growth rate (SGR) (Table 4).

Tissue enzyme activities. HUFA synthesis in liver microsomes, as measured by the recovery of radioactivity in the summed Δ6 and Δ5 desaturated products (18:4, 20:4, 20:5 and 22:5) of [1-^{14}C]18:3n-3, was significantly affected by both dietary oil content and CLA as determined by two-way ANOVA (Table 5). However there was significant interaction between the variables, as activity was significantly higher in fish fed 2% CLA compared to fish fed FO alone at the low oil level whereas the opposite trend was observed at the high oil level (Fig.1). HUFA synthesis activity in liver was significantly lower in fish fed the high oil diet (Table 5, Fig.1).

Dietary CLA did not significantly increase β-oxidation activity in white muscle irrespective of dietary oil content (Table 5, Fig.2). There was a trend suggesting CLA may also increase β-oxidation in red muscle in fish fed high dietary oil, but the high variation made this non-significant. Liver β-oxidation was significantly lower in fish fed the high oil diet, but dietary CLA had no effect on β-oxidation in liver (Table 5, Fig.2). Dietary oil content had no effect on β-oxidation activities in either muscle tissue.

CPT-I activity was significantly increased by dietary CLA in white muscle, and also in red muscle, particularly in fish fed the high oil diets (Table 5, Fig.3). CPT-I activity in white muscle was also significantly increased by high dietary oil. In contrast, hepatic CPT-I activity was unaffected by dietary oil content, but there was a trend for it to be decreased by dietary CLA in the low oil group (Table 5, Fig.3).

Tissue enzyme expression. The expression of fatty acid Δ6 and Δ5 desaturases in liver was significantly affected by both dietary CLA and oil content (Table 5). Specifically, expression of both desaturases was increased by dietary CLA and reduced by high dietary oil content (Fig. 4). CPT-I expression in all three tissues, red and white muscle, and liver, was significantly increased by dietary CLA (Table 5, Fig.5). However, CPT-I expression was not affected by dietary oil content in either muscle tissue although it was increased in liver tissue, at least in fish also being fed CLA (Table 5, Fig.5).

Tissue PPAR expression. The expression of all PPAR subtypes was significantly affected by both dietary CLA and oil content (Table 5). PPARα expression was increased by dietary CLA in liver and, to a lesser extent, white muscle (Fig.6). In red muscle, there was a strong interaction between dietary CLA and oil content such that CLA increased expression at low oil contents, but the opposite at high dietary oil content. High oil content may have induced increased expression of PPARα in red muscle although the interaction also made this difficult to interpret. PPARβ expression in the muscle tissues was similar to that for PPARα in these tissues. Thus the expression of PPARβ was increased by dietary CLA in white muscle whereas there was a significant interaction between CLA and dietary oil in red muscle, which made the effects of CLA difficult to interpret (Table 5). However, PPARβ expression was increased by high dietary oil, but dietary CLA decreased PPARβ expression in red muscle in fish fed high dietary oil (Fig.7). The expression of PPARβ in liver was greatest in fish fed 1% CLA (Table 5, Fig. 7). Expression of PPARγ in liver was significantly increased by dietary CLA, but dietary oil content had no significant effect (Table 5, Fig.8). PPARγ expression in muscle tissues was too low to be reliably determined.

DISCUSSION

CLA has the capacity to exert both agonistic and antagonistic effects on a wide variety of lipid metabolic factors. These effects are dependent upon the pattern of lipid metabolism, which itself varies with species, tissue type, and age, and dietary factors including lipid content, CLA isomer composition and duration of supplementation. Thus, it is difficult to define a specific biochemical mechanism of action. However, the view that CLA can affect lipid accumulation both by decreasing *de novo* fatty acid synthesis and increasing oxidation is well established, at least in mammals. For example, CLA inhibits fatty acid synthetase activity in rat liver (21), and suppresses TAG accumulation and increases fatty acid oxidation in 3T3-L1 adipocytes (22). It is hypothesised that CLA exerts such effects on lipid metabolism at the transcriptional level by altering gene expression of key regulatory proteins and enzymes mediated, in part, by PPARs. However, it was unclear whether CLA have the ability to alter lipid metabolism in fish in a manner similar to that of mammalian models (23).

In the present study, salmon fed CLA exhibited increased expression of Δ5 and Δ6 fatty acid desaturases in liver; particularly evident in the low oil diet, which displayed around a 2-fold increase in expression of Δ5 and Δ6 at 2% inclusion of CLA. This was at least partly reflected in HUFA synthesis activity, which increased in fish fed 2% CLA at the low dietary oil content. Analogously, Δ5 and Δ6 fatty acid desaturase expression was increased in mice fed CLA (24), whilst the amount of the HUFA, EPA and DHA, increased in hybrid striped bass *Morone chrysops, saxatilis* in response to dietary CLA (25). One explanation may be that transcription factors equivalent to mammalian PPARα and SREBP-1c are directly involved in regulating Δ6 and Δ5 desaturases in fish via a feedback mechanism. A similar mechanism for the regulation of desaturase gene expression has been proposed in mammals whereby Δ6 desaturase gene expression is partly regulated by PPARα (26). Thus, it was shown that ligand activation of PPARα via peroxisome proliferators may result in upregulation of Δ5 and Δ6 desaturase, whilst high PUFA feeding down regulated these genes in a SREBP-1-dependent mechanism (11). In salmon liver, CLA feeding was associated with increases in Δ5 and Δ6 desaturase

expression and HUFA synthesis in low oil treatments, which might be indicative of a role for ligand activation of PPARα by CLA. In contrast, in high oil diets, Δ5 and Δ6 expression and HUFA synthesis was repressed, even in the presence of similar amounts of CLA, possibly indicating a role for SREBP-1 proteins. Interestingly, CLA feeding also increased the levels of PPARα, providing some further support for this mechanism of regulation in salmon liver. Previously however, we reported that dietary CLA had no significant effect on either liver or flesh (muscle) fatty acid compositions in salmon, thus it appears that the increased fatty acid desaturase expression and HUFA synthesis activity does not have a major physiological consequence in terms of gross fatty acid compositions (16). That alterations in desaturase gene expression and HUFA synthesis activity in liver can have relatively little effect on tissue fatty acid compositions has been demonstrated previously in several trials in which salmon have been fed vegetable oils (see 27). The data from the present trial also show a correlation between desaturase expression and HUFA synthesis activity, and dietary oil content, with diets containing high oil conferring significantly lower levels of desaturase expression and HUFA synthesis activity compared to low oil diets. In addition to effects on HUFA synthesis, it is well established that there is a reduction in fatty acid synthesis (lipogenesis) as a result of high dietary oil levels, as demonstrated in Atlantic salmon and other fish species (28).

Beta-oxidation is the principal means of fatty acid catabolism in vertebrates and the basic mechanism of the pathway, and possibly regulation, appear to be highly conserved in mammals and fish (29). Recently, it has been suggested that CLA can increase mitochondrial β-oxidation in a variety of tissues in rats via an increase in CPT-I (30). Moreover, evidence of increased fatty acid oxidation after CLA supplementation is presented in similar studies, which indicated CLA increased gene expression and activity of CPT-I in mouse liver (31). However, in the present trial, β-oxidation capacity was not significantly affected in red or white muscle tissue of fish fed dietary CLA, despite mitochondrial β-oxidation predominating over peroxisomal β-oxidation in salmon red and white

muscle (32). In contrast, CLA significantly increased both CPT-I expression and activity in these tissues. The reasons for the lack of association of CPT-I with β-oxidation are unclear. Increased CPT-I activities in liver and red muscle of hamsters fed CLA was not correlated with increased fatty acid oxidation (33). The authors speculated this may have been due to increased sensitivity of CPT-I to malonyl-CoA in animals fed CLA (33). As in mammals, malonyl-CoA is an inhibitor of CPT-I in Atlantic salmon, but it is not known if CLA can affect its potency (34). There are few data in fish species to compare the present data with. However, dietary lipid level did not alter β-oxidation activity in the muscle of haddock *Melanogrammus aeglefinus L.* fed graded levels of oil (29). In the present study, dietary oil content had no major effect on β-oxidation activity or CPT-I mRNA levels in either red or white muscle, or CPT-I activity in red muscle, although CPT-I activity was increased by dietary oil content in white muscle.

Previous studies in rodents showed that CPT-I mRNA level was elevated in liver in response to dietary CLA (31). In the present study and similar to results in muscle, CPT-I gene expression was found to be upregulated in salmon liver in response to dietary CLA, although this was not accompanied by increased CPT-I or β-oxidation activities. Furthermore, CPT-I expression was higher in salmon liver in fish fed the high oil diet when fed in combination with CLA. Consistent with this it appeared that in fish fed CLA, CPT-I activity was lower in fish fed the low oil diet compared with fish fed the high oil. However, these effects on CPT-I expression and activity in liver were not reflected in β-oxidation activity, which surprisingly was reduced in fish fed the high oil diet. These results seem contradictory considering that the *in vivo* rate of fatty acid oxidation should be associated with *in vitro* CPT-I activity (35). Nevertheless, studies determining changes in energy metabolism in hamsters fed CLA also found disparities between the activity of CPT-I and lipid oxidation in the liver (33). Pertinent to this lack of correlation between CPT-I expression and activity and β-oxidation activity in liver is the fact that, with Atlantic salmon of similar age and size, catabolism of fatty acids in liver was

reported to be principally due to peroxisomal, rather than mitochondrial, β-oxidation (32). Consequently, CPT-I may not have a prominent role as a regulatory element of fatty acid oxidation in salmon smolt liver, considering it is primarily involved in mitochondrial metabolism (36). This may be relevant in the present study as it clearly provides a mechanism whereby changes in CPT-I expression and/or activity can be unrelated to β-oxidation activity in liver. This remains the case even considering that, in the present study, only total β-oxidation, combining mitochondrial and peroxisomal β-oxidation, was determined. Clearly though it also suggests that peroxisomal β-oxidation in salmon liver was unaffected by dietary CLA, and actually decreased by high dietary oil. In comparison, graded levels of dietary lipid showed no significant differences in β-oxidation activity in liver of haddock (29).

There is consolidating evidence that lipid homeostasis is, at least in part, modulated through the PPAR transcription factors in mammals. Recent studies suggest that CLA could mediate this activation by acting as high-affinity ligands for a number of PPAR isotypes, particularly PPARα (7). PPARα is intimately involved in the regulation of genes involved in mitochondrial fatty acid oxidative processes in mammals (37), whereas PPARγ is primarily involved in lipid deposition via preadipocyte differentiation and lipogenesis (38). Very recent work has elucidated that marine fish also share homologous gene sequences, with similar phylogenetic characteristics, to the mammalian PPAR counterparts, possibly also suggesting similar molecular roles (39,40). However, Atlantic salmon may possibly contain up to five PPAR genes, as opposed to three in mammals, and therefore the precise role of piscine PPARs have not been conclusively defined (40).

The effects of CLA on mammalian PPAR gene expression are ambiguous and seem to be dependant partly on species, partly on tissue type, and partly on CLA isomer. The majority of studies describe a decrease in PPARγ mRNA in isolated adipocytes, or in adipose tissue from mice treated with t-10, c-12 CLA or a 1:1 mixture of the two predominant CLA isomers (41,42). Conversely, CLA has also been reported to increase expression of PPARγ in liver of mice, and in white adipose tissue in rats

(43,44). Trans-10 c-12 CLA downregulated PPARα in mice (45), however there was no change in PPARα expression in liver of hamsters fed diets containing t-10 c-12 or c-9 t-11 CLA (46). In the present study, there was a trend for PPARα expression to be increased by dietary CLA in liver, red and white muscle of fish fed the low oil diets. In addition, upregulation of PPARα in response to dietary CLA was also observed in white muscle and liver in fish fed the high oil diets. As mentioned above, activation of PPARα (via peroxisome proliferators) can induce β-oxidation through upregulation of key enzymes such as CPT-I in mammals (37). The data in the present trial suggest that PPARα may also be implicated in the action of CLA. In general CLA increased the levels of both CPT-I mRNA and PPARα and in some cases PPARβ mRNA. The exceptions to this were PPARα and PPARβ expression in red muscle from fish fed high oil, which tended to decrease with dietary CLA inclusion, and at high CLA inclusion liver PPARβ was decreased. It has been shown in PPARα-knockout mice that the effects of CLA on body fat distribution and mitochondrial lipid catabolism genes are not mediated by PPARα, but that the peroxisomal β-oxidation gene acyl CoA oxidase is regulated by a PPARα-dependent mechanism (47). Acyl CoA oxidase shows high activity in salmon liver, but much lower activity in red muscle and very low activity in white muscle (34). Thus, it is possible that effects on mitochondrial lipid catabolic genes such as CPT-I could be mediated through PPARβ subtypes. However, PPARα expression exceeds that of PPARβ by an order of magnitude in muscle tissues suggesting PPARα would have the predominant regulatory role. Of course, it is not the absolute amount of PPAR that is important for target gene activation, but rather the binding of activating ligand.

Of the tissues investigated in salmon, PPARγ expression was only detected in liver. However, in agreement with work carried out on mice (43), PPARγ mRNA levels increased in liver of salmon fed CLA, and CLA have been reported to cause ‘fatty liver’ in mice (43). However, fish PPARγ does not share the same ligand activation profile as in mammals (40). Fish PPARγ does not respond to fatty acids and has specific amino acid differences compared to the mammalian form which explains lack of

activation, suggesting that is unlikely, despite increases in liver, that CLA would mediate its effects through this transcription factor. Indeed, neither liver size nor lipid content was increased in salmon fed CLA (16).

In summary, gene expression and activity of various lipid metabolic factors were altered in response to graded levels of CLA and/or dietary oil content in Atlantic salmon smolts. Specifically, some association was observed between dietary CLA, liver HUFA synthesis and desaturase gene expression, and liver PPARα expression although this varied with dietary oil content. In addition, some association between dietary CLA, CPT-I expression and activity, and PPARα expression was observed in muscle tissues. However, the magnitude of the changes in fatty acid metabolism observed were not sufficient to bring about major changes in the whole body lipid and fatty acid composition of the fish (16). In conclusion, this study has presented evidence that dietary CLA have effects on fatty acid metabolism in Atlantic salmon, but that there is little evidence of a direct mechanism involving PPAR transcription factors. However, considering the importance of dietary lipid in aquaculture, further work is required to assess the potential of CLA as a dietary supplement, and the role of PPARs in the regulation of lipid metabolism in fish.

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Legends to Figures

FIG. 1. Effects of dietary oil content and conjugated linoleic acid (CLA) on highly unsaturated fatty acid (HUFA) synthesis in the liver microsomes. Specific activities are presented as means ± SD (n = 3) and were determined as described in Materials and Methods.

FIG. 2. Effects of dietary oil content and conjugated linoleic acid (CLA) on total β-oxidation activities in tissue homogenates of red and white muscle, and liver. Specific activities are presented as means ± SD (n = 3) and were determined as described in Materials and Methods.

FIG. 3. Effects of dietary oil content and conjugated linoleic acid (CLA) on carnitine palmitoyltransferase I (CPT-I) activities in tissue homogenates of red and white muscle, and liver. Specific activities are presented as means ± SD (n = 3) and were determined as described in Materials and Methods.

FIG. 4. Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of Δ6 and Δ5 fatty acid desaturase genes in liver. Genes were determined by rtqPCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3).

FIG. 5. Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of carnitine palmitoyltransferase-I (CPT-I) in the liver, red and white muscle. Genes were determined by rtqPCR

and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3).

FIG. 6. Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of PPARα in the liver, red and white muscle, and adipose tissue. Genes were determined by rtqPCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3).

FIG. 7. Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of PPARβ in the liver, red and white muscle, and adipose tissue. Genes were determined by rtqPCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods.

FIG.8. Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of PPARγ in liver. Gene expression was determined by rtqPCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3).

Table 1

Formulations (percentage of dry ingredients) and proximate compositions (percentage of total diet) of experimental diets

	L0	L1	L2	H0	H1	H2
Fishmeal	44	44	44	50	50	50
Sunflower meal	15	15	15	4	4	4
Corn Gluten	8	8	8	8	8	8
Legume seeds	9	9	9	9	9	9
Cereal grains	10	10	10	0	0	0
Micronutrients	3	3	3	2	2	2
Fish oil	11	9.3	7.6	27	25.3	23.6
CLA	0	1.7	3.4	0	1.7	3.4
Moisture	8.0 ± 0.3	8.7 ± 0.1	8.9 ± 0.1	3.1 ± 0.1	4.2 ± 0.4	4.4 ± 0.1
Lipid	18.2 ± 0.3	17.4 ± 0.1	16.4 ± 0.5	33.2 ± 0.6	32.4 ± 1.2	32.6 ± 1.6
Protein	44.8 ± 0.1	44.8 ± 0.2	45.8 ± 0.5	47.0 ± 0.3	47.0 ± 0.3	47.1 ± 1.3
Ash	7.8 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	8.2 ± 0.1	8.1 ± 0.0	8.0 ± 0.2

Results for proximate compositions are means ± S.D. (n=3). Micronutrients, incl. essential amino acids (methionine and lysine), vitamins, minerals and astaxanthin (Carophyll pink®), Biomar A/S, Brande, Denmark. H0, H1 and H2, diets containing fish oil at 34% and supplemented with 0, 1 and 2% CLA; L0, L1 and L2, diets containing fish oil at 17% and supplemented with 0, 1 and 2% CLA.

Table 2

Fatty acid composition (percentage of weight) of experimental diets containing conjugated linoleic acid (CLA) fed to Atlantic salmon (*Salmo salar*).

	L0	L1	L2	H0	H1	H2
Total saturated	30.7 ± 1.0 ^{ab}	28.6 ± 1.1 ^{b}	28.9 ± 1.4 ^{b}	31.9 ± 0.4 ^{a}	30.6 ± 0.7 ^{ab}	31.0 ± 0.8 ^{ab}
Total monoenes	30.1 ± 0.7	30.0 ± 0.7	31.0 ± 0.9	29.8 ± 0.6	29.3 ± 0.4	30.2 ± 0.5
18:2n-6	6.4 ± 0.2 ^{b}	6.6 ± 0.1 ^{ab}	6.8 ± 0.2 ^{a}	3.1 ± 0.1 ^{c}	3.3 ± 0.0 ^{c}	3.2 ± 0.0 ^{c}
20:4n-6	0.9 ± 0.0 ^{ab}	0.8 ± 0.0 ^{bc}	0.7 ± 0.0 ^{c}	1.0 ± 0.1 ^{a}	0.9 ± 0.0 ^{ab}	0.8 ± 0.0 ^{bc}
Total n-6 PUFA[1]	8.4 ± 0.1 ^{a}	8.2 ± 0.5 ^{a}	8.2 ± 0.6 ^{a}	5.1 ± 0.1 ^{b}	5.0 ± 0.2 ^{b}	4.8 ± 0.5 ^{b}
18:3n-3	1.2 ± 0.0 ^{a}	1.1 ± 0.0 ^{ab}	1.0 ± 0.0 ^{a}	1.2 ± 0.0 ^{a}	1.2 ± 0.0 ^{a}	1.1 ± 0.0 ^{ab}
18:4n-3	2.5 ± 0.1 ^{b}	2.1 ± 0.0 ^{d}	1.8 ± 0.0 ^{e}	2.7 ± 0.0 ^{a}	2.6 ± 0.0 ^{ab}	2.3 ± 0.0 ^{c}
20:4n-3	0.7 ± 0.0 ^{ab}	0.6 ± 0.0 ^{bc}	0.5 ± 0.0 ^{c}	0.8 ± 0.0 ^{a}	0.8 ± 0.0 ^{a}	0.7 ± 0.0 ^{ab}
20:5n-3	11.8 ± 0.7 ^{b}	10.2 ± 0.2 ^{c}	8.4 ± 0.5 ^{d}	13.6 ± 0.1 ^{a}	13.0 ± 0.1 ^{a}	11.5 ± 0.1 ^{b}
22:5n-3	1.8 ± 0.1 ^{b}	1.6 ± 0.0 ^{c}	1.3 ± 0.1 ^{d}	2.0 ± 0.0 ^{a}	1.9 ± 0.0 ^{a}	1.7 ± 0.0 ^{bc}
22:6n-3	12.7 ± 0.9 ^{a}	11.5 ± 0.5 ^{b}	9.5 ± 0.1 ^{c}	12.9 ± 0.0 ^{a}	12.4 ± 0.2 ^{a}	10.9 ± 0.1 ^{b}
Total n-3 PUFA[2]	30.8 ± 1.8 ^{a}	27.3 ± 0.8 ^{b}	22.6 ± 0.7 ^{c}	33.2 ± 0.3 ^{a}	31.8 ± 0.4 ^{a}	28.2 ± 0.2 ^{b}
CLA (9c,11t)	0.0 ± 0.0 ^{d}	2.9 ± 0.5 ^{b}	4.8 ± 1.6 ^{a}	0.0 ± 0.0 ^{d}	1.6 ± 0.2 ^{c}	2.9 ± 0.7 ^{b}
CLA (10t,12c)	0.0 ± 0.0 ^{d}	3.0 ± 0.4 ^{b}	4.6 ± 1.7 ^{a}	0.0 ± 0.0 ^{d}	1.7 ± 0.4 ^{c}	2.9 ± 0.8 ^{b}
Total CLA	0.0 ± 0.0 ^{d}	5.9 ± 0.5 ^{b}	9.4 ± 1.5 ^{a}	0.0 ± 0.0 ^{d}	3.3 ± 0.4 ^{c}	5.8 ± 0.7 ^{b}
Total PUFA	39.2 ± 1.7	41.5 ± 1.8	40.2 ± 2.4	38.3 ± 0.2	40.13 ± 1.1	38.8 ± 1.3

Values are means ± SD of 3 samples. Significance of differences between mean values were determined by one-way ANOVA followed, where appropriate, by Tukey's multiple comparison test as described in the Materials and methods. Values within a row with a different superscript letter are significantly different ($P < 0.05$).[1], totals include 18:3n-6, 20:2n-6 and 22:5n-6 present in some samples at up to 0.4%; [2], totals contain 20:3n-3 present at up to 0.2%. H0, H1 and H2, diets containing fish oil at 34% and supplemented with 0, 1 and 2% CLA; L0, L1 and L2, diets containing fish oil at 17% and supplemented with 0, 1 and 2% CLA. PUFA, polyunsaturated fatty acids.

Table 3

Forward (sense) and reverse (antisense) primers used for real-time quantitative RT-PCR

Gene	PCR product length	Forward primer	Reverse primer
Δ5 Desaturase	192	5’-GTGAATGGGGATCCATAGCA-3’	5’-AAACGAACGGACAACCAGA-3’.
Δ6 Desaturase	181	5'-CCCCAGACGTTTGTGTCAG-3’	5’-CCTGGATTGTTGCTTTGGAT-3’
CPT-1	161	5'-GAGAGAGCTGCGACTGAAAC-3'	5'-GACAGCACCTCTTTGAGGAA-3'
PPARα	204	5'-ATCTTCCACTGCTGCCAGTGC-3'	5'-GATGAAGCCCGATCCGTAGGCCACCAGG-3'
PPARβ1	517	5'-TACCGCTGCCAGTGCACCACGGTG-3'	5'-TTCTGGACCAAGCTGGCGTTCTCA-3'
PPARγ	266	5'-TATCTCCCCTCTCTAGAGTA-3'	5'-AGGGCTTATCGTTTACTGAACCTTGATACACGC-3'

Table 4

Final weights of Atlantic salmon (*Salmo salar*) fed diets containing conjugated linoleic acid (CLA)

Diet	Initial Weight (g)	Final Weights (g)	SGR
L0	87.8 ± 1.5	256.3 ± 18.7	1.31
L1	87.4 ± 0.5	236.3 ± 17.6	1.21
L2	87.9 ± 2.2	236.7 ± 18.4	1.21
H0	85.5 ± 1.5	245.4 ± 12.1	1.29
H1	89.3 ± 0.9	260.8 ± 13.1	1.31
H2	86.9 ± 0.6	260.4 ± 5.4	1.34

Data are presented as means ± SD, n = 100 and 10 for initial and final weights); SGR, specific growth rate (%/day) = 100 x [(ln $weight_{final}$ - ln $weight_{initial}$) x $days^{-1}$]; L0, L1 and L2, low lipid diets with 0, 1 and 2% CLA; H0, H1 and H2, high lipid diets with 0, 1 and 2% CLA. There were no significant differences between dietary treatments as determined by two-way ANOVA.

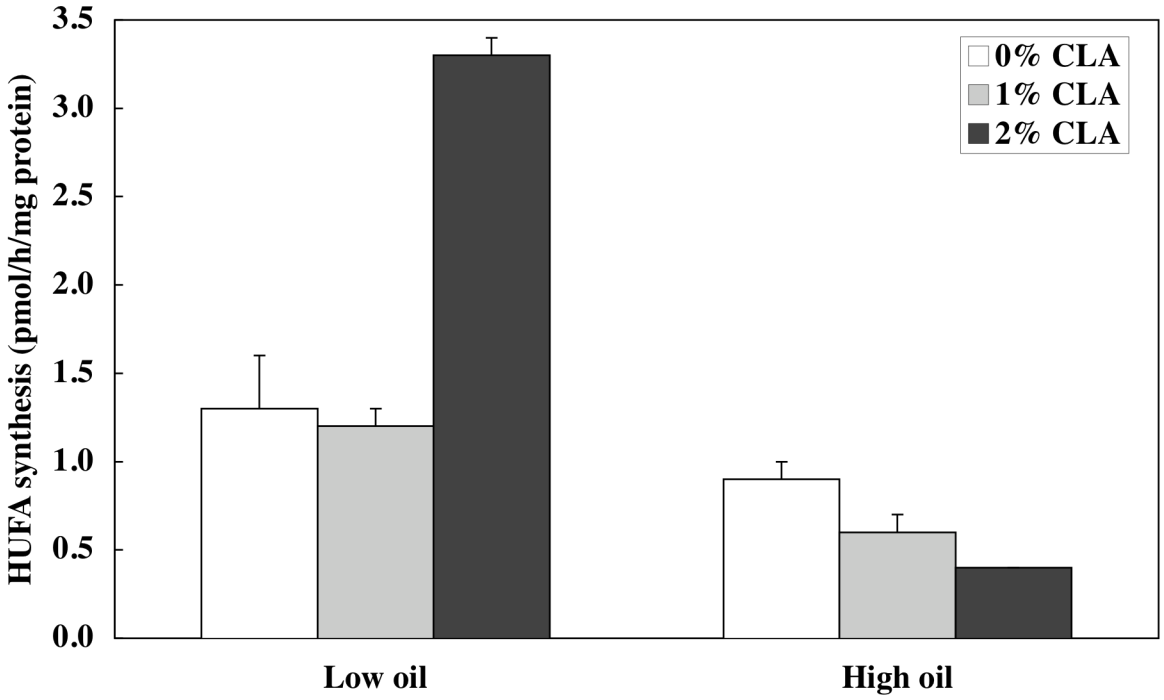
Table 5

Significance (P values) of effects of dietary conjugated linoleic acid (CLA) and oil content (Oil), and their interaction (Interact.) as determined by two-way ANOVA as described in the Methods section.

	P Value		
	CLA	Oil	Interact.
Liver			
HUFA synthesis activity	**< 0.0001**	**< 0.0001**	**< 0.0001**
Δ6 desaturase expression	**0.0009**	**<0.0001**	**0.0156**
Δ5 desaturase expression	**0.0031**	**0.0009**	0.5247
β-oxidation activity	0.2757	**0.0136**	0.5943
CPT-I activity	0.8506	0.5573	0.8676
CPT-I expression	**0.0027**	**0.001**	**0.0483**
PPARα expression	**0.0001**	**0.0347**	0.1144
PPARβ expression	**0.0004**	**0.0445**	0.1649
PPARγ expression	**0.0449**	0.2061	0.237
Red muscle			
β-oxidation activity	0.8133	0.3457	0.5257
CPT-I activity	**0.0355**	0.1511	0.4857
CPT-I expression	**0.0022**	0.7695	0.3469
PPARα expression	0.2603	**0.0172**	**0.0034**
PPARβ expression	0.1182	**<0.0001**	**0.046**
White muscle			
β-oxidation activity	0.0795	0.1473	0.8623
CPT-I activity	**0.0075**	**0.0001**	0.1409
CPT-I expression	**0.0077**	0.7394	0.2716
PPARα expression	**0.0292**	0.0518	0.0836
PPARβ expression	**0.0032**	**0.0006**	0.0822

CPT-I, carnitine palmitoyltransferase-I; HUFA, highly unsaturated fatty acids; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acid.

Fig.1


HUFA synthesis (pmol/h/mg protein)
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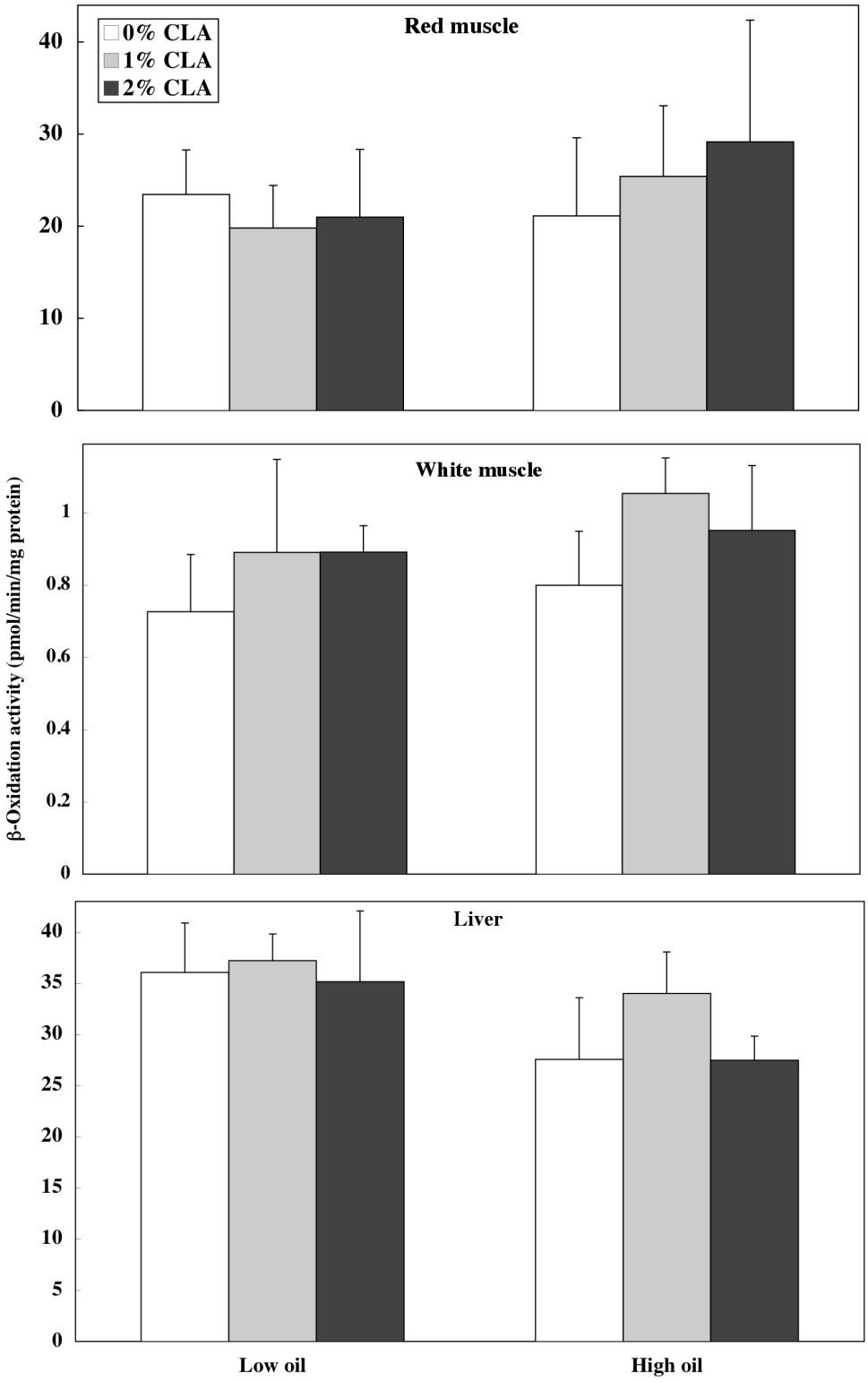
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0% CLA
1% CLA
2% CLA
Low oil
High oil


Red muscle
0% CLA
1% CLA
2% CLA
40
30
20
10
0
White muscle
1
0.8
0.6
0.4
0.2
0
β-Oxidation activity (pmol/min/mg protein)
Liver
40
35
30
25
20
15
10
5
0
Low oil
High oil

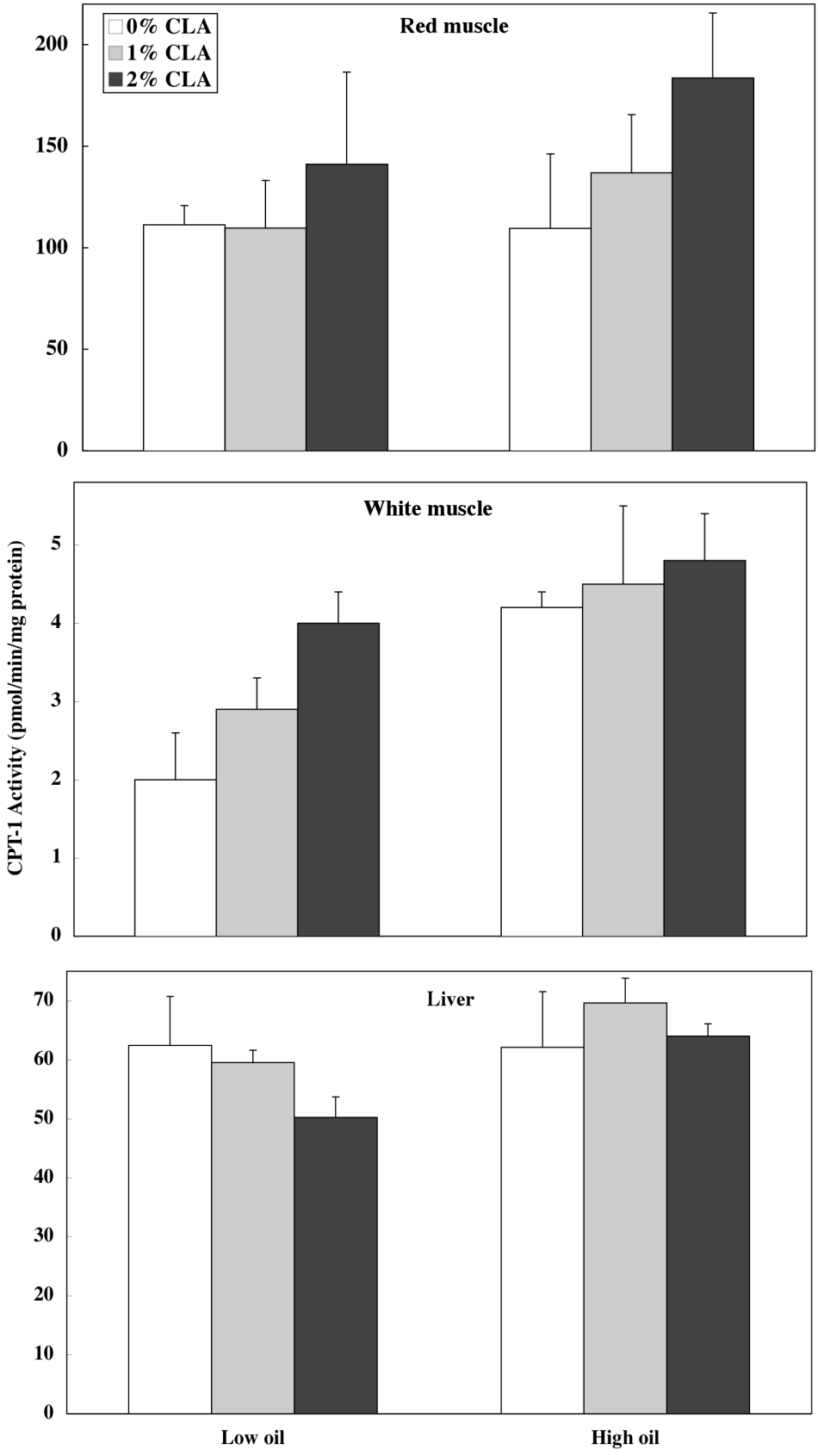
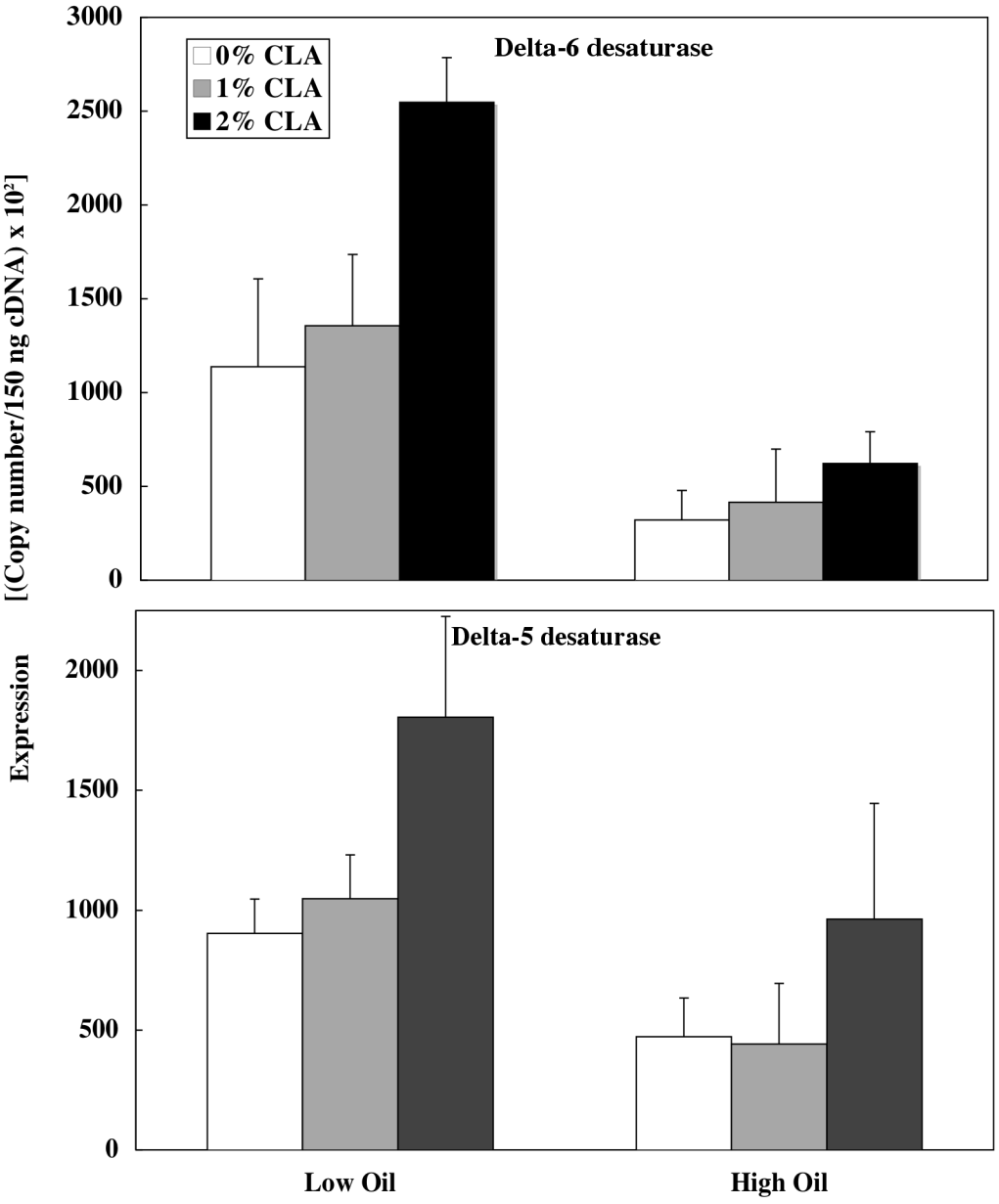

0% CLA
1% CLA
2% CLA
Red muscle
White muscle
Liver
CPT-1 Activity (pmol/min/mg protein)
200
150
100
50
0
5
4
3
2
1
0
70
60
50
40
30
20
10
0
Low oil
High oil

Fig.4


Delta-6 desaturase
0% CLA
1% CLA
2% CLA
[(Copy number/150 ng cDNA) x 10²]
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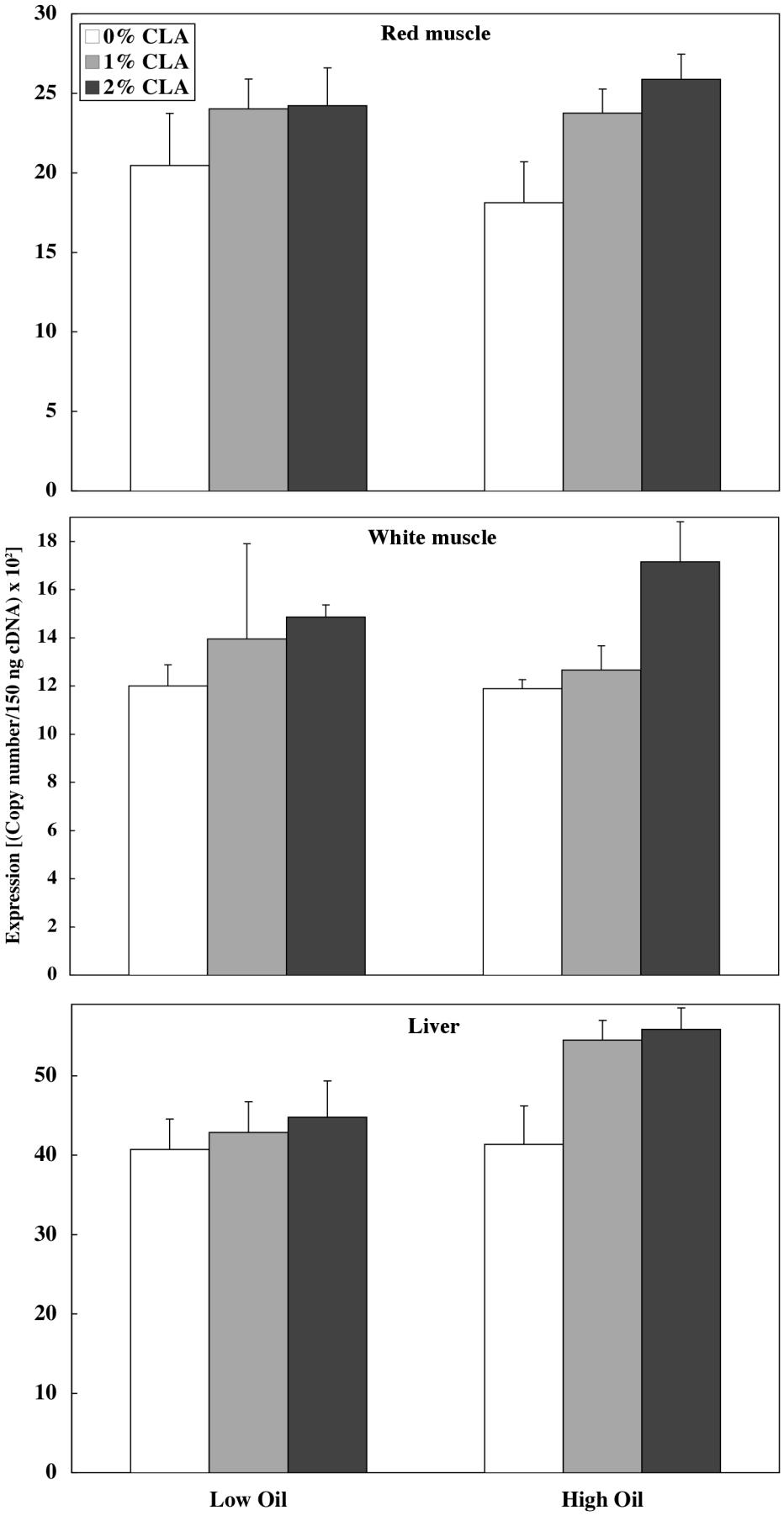
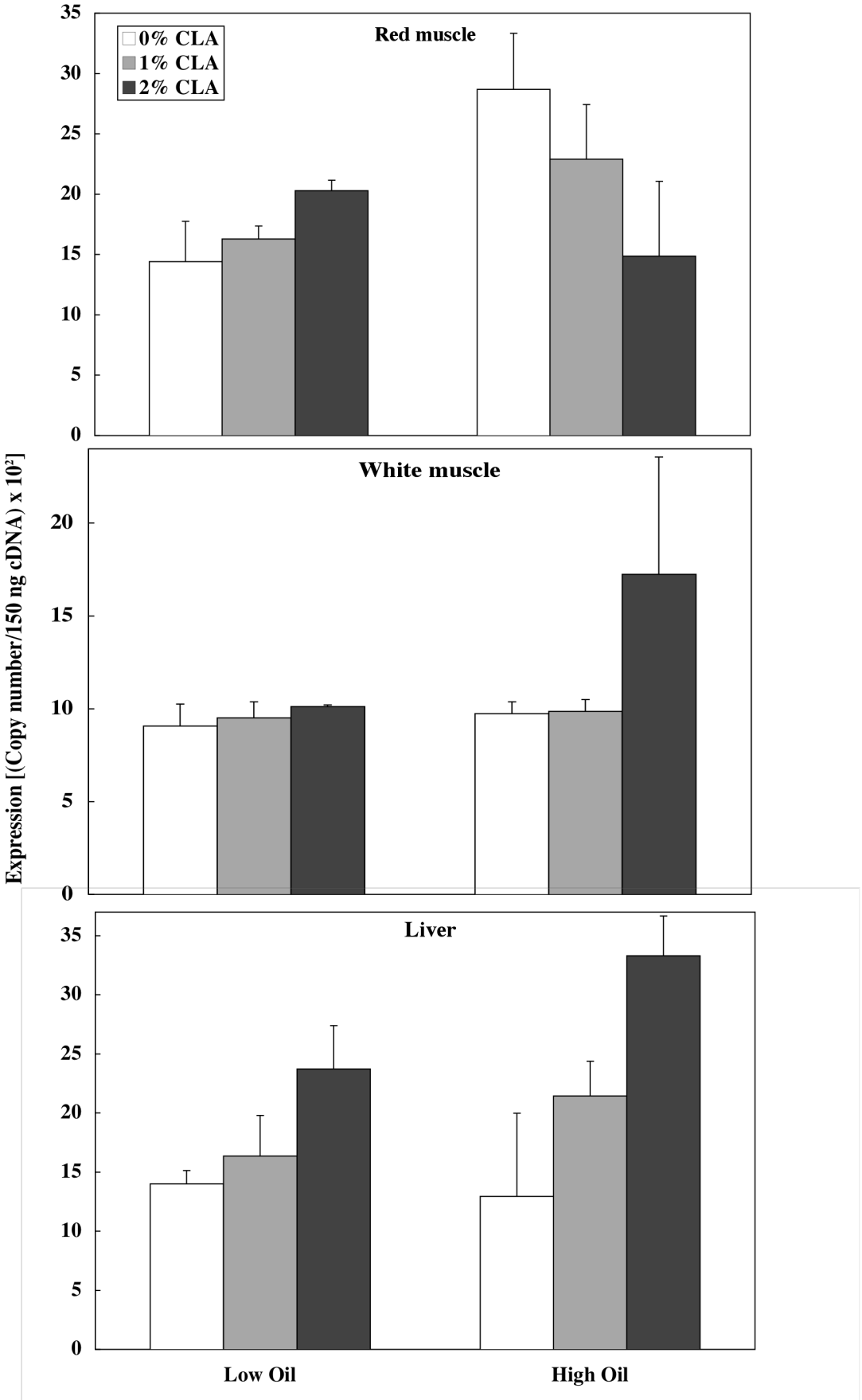
2500
2000
1500
1000
500
0
Delta-5 desaturase
Expression
2000
1500
1000
500
0
Low Oil
High Oil


0% CLA
1% CLA
2% CLA
Red muscle
White muscle
Liver
Expression [(Copy number/150 ng cDNA) x 10²]
Low Oil
High Oil


Red muscle
0% CLA
1% CLA
2% CLA
White muscle
Liver
Expression [(Copy number/150 ng cDNA) x 10²]
Low Oil
High Oil

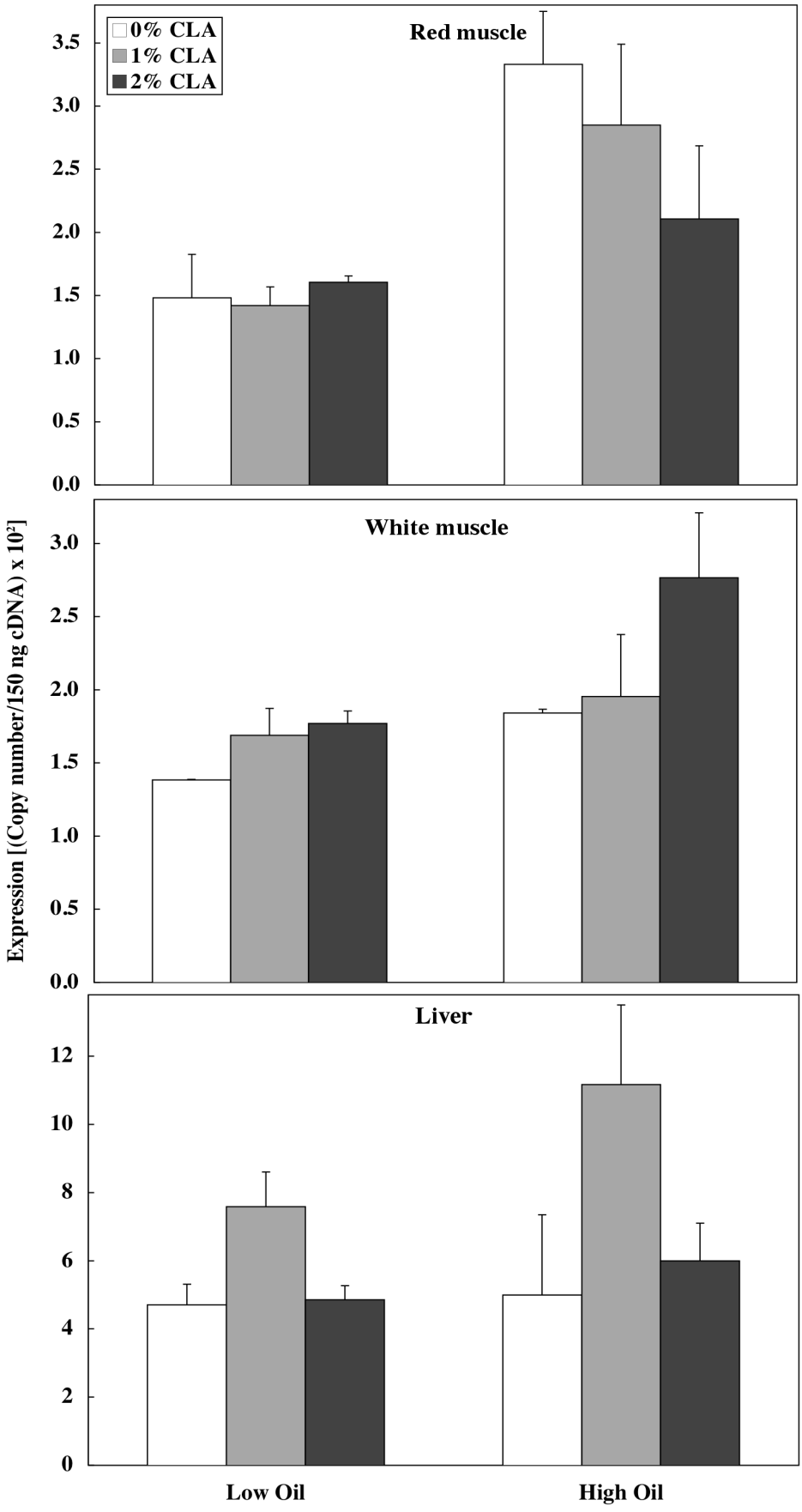
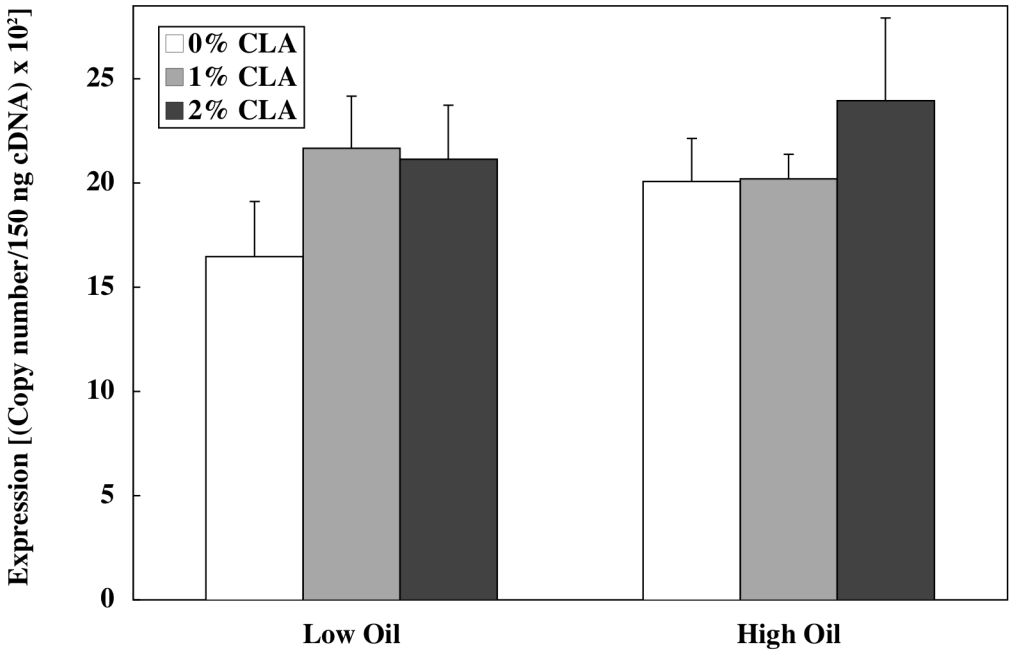

0% CLA
1% CLA
2% CLA
Red muscle
White muscle
Liver
Expression [(Copy number/150 ng cDNA) x 10³]
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
2
4
6
8
10
12
Low Oil
High Oil

Fig.8


0% CLA
1% CLA
2% CLA
Expression [(Copy number/150 ng cDNA) x 10²]
25
20
15
10
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Low Oil
High Oil