

Body growth, mitochondrial enzymatic capacities and aspects of the antioxidant system and redox balance under calorie restriction in young turbot (*Scophthalmus maximus*, L.)

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Abstract

Calorie restriction (CR) without undernutrition has been found to enhance stress resistance and life span in endotherms and ectotherms. We investigated the effect of 30% reduction in food offering on growth, aerobic capacities and oxidative stress parameters in young turbot (*Scophthalmus maximus*, L.).

No differences in body weight, length and hepatosomatic index between the *ad libitum* (AL)-fed and the CR group occurred after 55 days of diet application. Of the mitochondrial marker enzymes, only citrate synthase (CS) activity in the liver was reduced under CR, whereas muscle CS activity and cytochrome oxidase (CCO) activity in both tissues remained the same in both feeding groups. The concentration of reduced glutathione increased significantly during feeding in muscle of CR fish, resulting in a more reduced glutathione redox ratio (GSH/GSSG) compared with AL fish muscle. Thiobarbituric acid-reactive lipid hydroperoxides (lipid peroxidation) but not protein carbonyl content (protein oxidation) was significantly reduced in CR fish muscle. Liver oxidative stress parameters did not vary significantly between experimental feeding groups.

We conclude that 30% CR over 8 weeks has no adverse effect on young turbot. On the contrary, CR supports a reduced tissue oxidation state and reduces accumulation of lipid peroxidation products in muscle at sustained muscular aerobic capacity.

Keywords: turbot, calorie restriction, mitochondria, glutathione, oxidative stress

Introduction

Growth performance in response to diet of commercially cultured fish species, such as turbot (*Scophthalmus maximus*, L.), is of great concern to understand the production cycle, to ease management, and to calculate costs in aquaculture. Fish feeds are usually the largest variable cost item in an intensive culture system (Shepherd & Bromage 1988). Intensively farmed fish require high-protein feeds (Randall, Bolis & Agradi 1990), which originate mainly from fishmeal (Mock, White & Wagener 2001). Owing to the global use of fishmeal and the increasing demand from aquaculturists, the prices of fishmeal are increasing. In this context, it could become important to improve feeding and stocking management.

Because of the high variation in food availability in natural aquatic systems, fish are adapted to survive extended periods of starvation. Variable degrees of starvation during ontogeny lead to variable growth rates (Morales, Perez-Jimenez, Hidalgo, Abellán & Cardenete 2004). In several cold and warm water fish species, phases of rapid growth follow slow growth during food restriction, a so-called compensatory growth phase (Zhu, Cui, Ali & Wootton 2001; Skalski, Picha, Gilliam & Borski 2005). Moreover, it is debated whether *ad libitum* (AL) feeding, as it is practiced in aquaculture to ensure high production output, is healthy for the cultured organisms. Studies with AL-fed primates suggest that this is not the case (Bodkin, Alexander, Ortmeier, Johnson & Hansen 2002; Lamming, Wood & Sinclair 2004). Gamperl and Farrell (2004) report fish to be frequently overfed

under aquaculture conditions with ensuing fat depositions around the heart, cardiac deformities and arteriosclerosis. It can be inferred that these fish, if kept alive to await natural death, would have a shorter life expectancy than wild fish in the absence of predators.

Calorie restriction (CR) is defined as a dietary regime low in calories without undernutrition (Koubova & Guarente 2003). Calorie restriction is the only scientifically established means by which a substantial extension of maximum life span can be achieved in most, if not all, heterotrophic organisms tested (Sohal & Weindruch 1996, also reviewed in Warner & Starke-Reed 1997). This is partly explained by a reduction in the ad-hoc production of reactive oxygen species (ROS) by (isolated) mitochondria in CR animals, which can decline to less than half that of non-CR animals (Warner & Starke-Reed 1997; Weindruch, Keenan, Carney, Fernandez, Feuers, Floyd, Halter, Ramsey, Richardson, Roth & Spindler 2001). The reduction in ROS formation is not simply a consequence of lower respiration rates, as CR, after an initial adaptive phase, does not reduce steady-state metabolic rates (Koubova & Guarente 2003). Instead, either less ROS are formed by mitochondria during CR, or higher antioxidant protection, including the tissue redox buffer glutathione, is achieved under limited calorie intake. In rodents, CR causes a reduction in the average daily body temperature (Duffy, Feuers, Leakey, Nakamura, Turturro & Hart 1989), which may accentuate the diet-induced slow-down of mitochondrial ROS production.

Cellular production of ROS is inversely correlated with maximum life span at the intra-specific level, and even in inter-specific comparisons (Ku, Brunk & Sohal 1993). However, the control of mass-specific ROS formation is only one facet of the life-prolonging effect of CR. Characteristic changes during steady-state CR are neuroendocrine changes of the insulin/IGF-1 signalling pathway, a more intensive turnover of body protein after depletion of glycogen reserves and changes in gene expression (for a review, see Weindruch *et al.* 2001; Koubova & Guarente 2003; Stuart & Brown 2006). The overall strategy during CR seems to be divergence of energy allocation from fecundity to body maintenance and stress resistance. Many invertebrates, invertebrate larvae and laboratory rodents show enhanced resistance to a range of stresses and prolonged life expectancy under CR (Kirkwood & Austad 2000; Weindruch *et al.* 2001; Stuart & Brown 2006).

Several studies investigated the effect of calorie restriction and starvation on the metabolic shift from lipogenesis to β -oxidation of fatty acids in cold water fish (Guderley, Lapointe, Bédard & Dutil 2003; Martínez, Guderley, Dutil, Winger, He & Walsh 2003). Few papers, so far, have included oxidative stress parameters (Guderley *et al.* 2003; Pascual, Pedrajas, Toribio, Lopez-Barea & Peinado 2003; Morales *et al.* 2004). Moreover, the reports are difficult to compare because different parameters were analysed and food limitation was applied in different ways in the different papers.

In the present study, we exposed young turbot to a calorie-restricted diet (30% less than AL) to examine how young, rapidly growing stages respond to CR. We investigated the effects on white muscle, which represents a major proportion of body mass, and is functionally important for sustained swimming activity, and, in the liver, the metabolically most active and storage tissue. Specifically, we were interested in observing whether body growth was retarded in the short term and whether or not mitochondrial enzymatic capacities [cytochrome c oxidase (CCO) and citrate synthase (CS)] were retained in CR turbot. We examined the changes in fatty acid composition in the liver to observe whether lipid stores were stocked with dietary lipids during CR feeding. Additionally, two oxidative stress parameters, protein carbonylation and formation of thiobarbituric acid-reactive lipid hydroperoxides (TBARS), were quantified in CR and AL fish as markers for oxidative damage of proteins and lipids. The glutathione status (reduced GSH: oxidized GSSG) was measured to examine whether CR fish were able to stabilize the cellular redox environment, and whether or not reduced glutathione also plays a role as an antioxidant.

Scrutinizing the approach of calorie restriction for young turbot, the results from this study may provide valuable information for future management concepts in aquaculture. If the approach of AL feeding leads to surfeit turbot with high-calorie food, this may eventually impinge on the profitability of fish farming.

Materials and methods

Origin of experimental animals and holding unit

In this experiment, juvenile turbot ($n = 170$, length: $11 \text{ cm} \pm 0.66$), reared at Venø fish farm, Struer, at the Limfjord in Denmark, were shipped to the Alfred

Wegener Institute on 24 May 2004. The fish were maintained at 15 °C (± 0.7) water temperature in a common recirculation system. The process water was treated by a typical cleaning unit containing a hydrocyclone for particle clearance, a biofilter to oxidize ammonium (NH_4^+) and an ozone-driven protein skimmer. As no de-nitrification of the water was performed, parts of the process water (about 20% of the total volume) needed to be exchanged at regular intervals. Light was provided using a 36 W fluorescent daylight tube at a 12:12 light/dark cycle. Water in the holding tanks (87.5 × 78 cm, 150 L) was kept aerated (>80% saturation), and the salinity was adjusted with artificial salt (Tropic Marin[®], Dr Biener GmbH) to 25.3 ‰ (± 0.5). Twice a day, fish were fed with dry pellets of about 4 mm diameter (DAN-EX 1562, Bio-Mar, Brande, Denmark; 58% protein, 15% fat and 10.5% carbohydrate). The remaining food pellets and fish faeces were removed from the tanks when fish stopped eating.

Experimental set up for the feeding experiment

Untagged fish ($n = 170$) were evenly distributed into two separate tanks and were maintained without feeding for 2 weeks until the start of the experiment. Inorganic nutrient levels (NH_4^+ , NO_2^- , NO_3^-) were recorded daily using photometric test systems (Hach-Lange, Düsseldorf, Germany) and water was exchanged when limit values were reached (NH_4 : 0.5 mg L^{-1} , NO_2 : 0.5 mg L^{-1} and NO_3 : 100 mg L^{-1}). At no time did NH_4^+ and NO_2^- levels exceed the limit values; however, frequent water exchanges were necessary to maintain a low NO_3^- concentration. pH was controlled daily and adjusted to between 7.5 and 8.2 using calcium hydroxide.

At the beginning of the experiment, fish were fed 2% of the overall biomass twice a day at 10:00 and 15:00 hours. On 8 July 2004, the mean weight of experimental fish amounted to $23.06 \pm 0.66 \text{ g}$ and the mean length to $11.46 \pm 4.77 \text{ cm}$ ($n = 36$ subsample). Starting on 8 July 2004, one group of fish ($n = 85$) was fed AL group twice per day. Net consumption of pellets in the AL group was calculated as offered pellet amount minus the amount rejected by the fish. The remaining fish ($n = 85$) represent the CR group because they received 30% less pellet weight than the AL group, on each day. This feeding experiment lasted 2 months. The weight and length of 10 randomly collected individuals from each group were

measured every 2 weeks. The weight and length recordings of 36 randomly picked individuals from both feeding groups were carried out on 13 September 2004 at the end of the experiment.

Animals for biochemical analyses were killed by a cut through the spine and immediately dissected. The liver and a piece of white muscle were freeze-clamped in liquid nitrogen and kept at -80 °C until analysis. Before freezing, liver weight was recorded. To do so, the liver was transiently stored on ice. The time between sampling and freezing was $< 1 \text{ min}$.

Mitochondrial enzymes

For measurements of mitochondrial enzymes cytochrome-c oxidase (CCO: E.C. 1.9.3.1, end oxidase of respiratory chain) and citrate synthase (CS: E.C. 4.1.3.7, enzyme of the citric acid cycle), frozen samples of liver and muscle were ground in liquid nitrogen and homogenized with a glass homogenizer (Nalgene, Fisher Scientific, Pittsburgh, PA, USA) in Tris-HCl buffer [20 mM Tris-HCl, 1 mM EDTA, 0.1% (v/v) Tween[®] 20, pH 7.4] 1:8 (w/v). For CCO measurements, homogenates were centrifuged for 10 min at 1000 *g* and 0 °C. The CCO activity was determined according to Moyes, Mathieu-Costello, Tsuchiya, Filburn and Hansford (1997) by measuring the oxidation of reduced cytochrome c at 550 nm in 20 mM Tris-HCl buffer with 0.5% Tween 20, pH 8.0. Fully reduced cytochrome c was prepared by reducing a 1 mM stock solution with sodium di-thionide in phosphate buffer (10 mM KPi, pH 7.0 with 1 mM EDTA). The buffer was previously purged with N_2 . Dithionide was removed using a Sephadex G-25 absorber column over which the cytochrome c solution was filtered through centrifugation at 2000 *g* for 2 min at 0 °C. Reduced cytochrome c was collected into brown flasks and could be stored frozen for at least 4 weeks. Activity was calculated using the millimolar extinction coefficient $\epsilon_{550} \text{ mM}^{-1} \text{ cm}^{-1}$.

Homogenates for CS activity were sonicated for 15 min in a Branson Sonifier 450 (Branson, Danburg, CT, USA) at output control 8, Duty cycle 50% cooled to 0 °C and centrifuged at 7400 *g* for 5 min at 0 °C. The CS activity was measured according to the method of Sidell, Driedzic, Stowe and Johnston (1987) recording the absorbance increase of 5 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] in 100 mM Tris-HCl (pH 8.0), 20 mM Acetyl-CoA and 20 mM Oxaloacetat at 412 nm. Activity was calculated using the millimolar extinction coefficient $\epsilon_{412} \text{ mM}^{-1} \text{ cm}^{-1}$.

Oxidative stress parameters

For protein carbonylation (after Levine, Garland, Oliver, Amici, Climent, Lenz, Ahn, Shaltiel & Stadtman 1990), tissue samples were homogenized in 50 mM HEPES buffer, pH 7.4, containing 125 mM KCl, 1.25 mM EDTA, 0.6 mM MgSO₄ and protease inhibitors (0.5 µg mL⁻¹ leupeptine, 0.7 µg mL⁻¹ pepstatin, 40 µg mL⁻¹ phenylmethylsulphonyl fluoride, 0.5 µg mL⁻¹ aprotinin) and centrifuged at 100 000 *g* for 15 min at 4 °C. Supernatants were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) dissolved in 2 M HCl to allow DNTP to bind to the carbonyl groups. Blanks were run with HCl only. Afterwards, proteins were precipitated with 0.2 mL 100% trichloroacetic acid (TCA) and centrifuged for 10 min at 11 000 *g*. The protein pellet was washed three times with ethanol:ethylacetate (1:1), resuspended in guanidine hydrochloride (6 M in 20 mM potassium phosphate, pH = 2.5) and incubated at 37 °C until complete resuspension. The carbonyl content was measured spectrophotometrically in the resulting suspension at 360 nm (molar extinction coefficient $\epsilon = 22\,000\text{ M}^{-1}\text{ cm}^{-1}$). To relate carbonyl content to the amount of extracted proteins in each sample, the protein content was subsequently measured with the method of Bradford (1976).

The TBARS concentrations were determined according to the method of Uchiyama and Mihara (1978). Deep-frozen tissues were ground and homogenized in 1:10 (w/v) 0.2% H₃PO₄. 0.4 mL of homogenate was mixed with 0.4 mL of 1% thiobarbituric acid (TBA). Individual blanks were prepared by replacing the TBA solution with 3 mM HCl. The pH was adjusted to 1.6. The samples were heated to 100 °C for 1 h. After cooling to room temperature, 2 mL butanol was added to the samples and blanks. After mixing for 40 s, the butanol phase was separated by centrifugation for 5 min at 14 000 *g* and the concentration of the lipidhydroperoxide–TBA acid complex in solution was measured as the difference in absorbance between 532 and 600 nm. The concentration was quantified from a previously obtained calibration curve.

Determination of reduced (GSH) and oxidized (GSSG) glutathione by high-performance liquid chromatography (HPLC)

The content of GSH and GSSG was determined according to the method of Fariss and Reed (1987). Fro-

zen tissue of muscle and liver was ground in liquid nitrogen and the resulting powder was homogenized in 1:10 (w:v) pre-cooled PCA (10% containing 2 mM bathophenanthroline-disulphonic acid). After centrifugation at 15 000 *g* for 5 min at 4 °C, 500 µL of the supernatant was mixed with 10 µL pH indicator [1 mM *m*-cresol purple sodium salt containing 0.5 M iodoacetic acid (IAA)]. Fifty microlitres 1 mM γ -glutamyl-glutamate (in 0.3% PCA) was added as an internal standard. The pH was adjusted to 8.5 with 5 M KOH (containing 0.3 M *N*-morpholine-propanesulphonic acid). The mixture was incubated at room temperature for 45 min, to allow IAA to bind GSH. Subsequently, samples were centrifuged for 5 min at 15 000 *g* and 4 °C. Three hundred microlitres of the supernatant was added to double the amount of 1% 1-fluor-2,4-dinitrobenzene (diluted in 100% ethanol, HPLC grade) and derivatized in dark vials at room temperature over 24 h. After centrifugation at 7500 *g* for 1 min at 4 °C and filtration through 0.2 µm nylon membrane filters, samples were stored in dark HPLC vials at – 20 °C.

The HPLC determination was carried out on a Beckmann Coulter HPLC System using a NH₂ – spherisorp column, 5 µm 240 × 4 mm (Waters, Germany). Solvent A: 80% methanol and solvent B: sodium acetate stock in 80% methanol (20:80). Sodium acetate stock was prepared by dissolving 500 g Na-acetate in 224 mL Milli-Q water and 695 mL of concentrated HPLC-grade acetic acid. The gradient programme consisted of a 10-min hold at 90% A, followed by a 25-min linear gradient to 25% A at a flow rate of 1 mL min⁻¹ and 2.3–2.8 kpsi back-pressure. Peaks were recorded with a photodiode array detector at 365 nm.

Lipid analyses

Liver sample dry mass was determined after lyophilization for 48 h and then total lipids were extracted according to the method of Folch, Lees and Sloane Stanley (1957). The lipid content was determined gravimetrically and expressed as total lipid mass (mg) and in per cent of dry mass (% DM). The fatty acid composition of total lipids was determined by gas chromatography (GC), without differentiation of individual lipid classes (Kattner & Fricke 1986). Briefly, an aliquot of the total lipid extract was hydrolysed in methanol containing 3% concentrated sulphuric acid, and fatty acids were converted to methyl esters (FAME) by transesterification at 80 °C

for 4 h. After extraction with hexane, FAME were analysed with a gas liquid chromatograph (Agilent 6890) on a 30 m × 0.25 mm i.d. wall-coated open tubular column (film thickness: 0.25 µm, liquid phase: DB-FFAP) using temperature programming (Kattner & Fricke 1986; Graeve, Albers & Kattner 2005). The fatty acids were identified using standard mixtures and, if necessary, additional confirmation was performed by GC–MS. Fatty acids were grouped according to their presumed origin: the 16:0, 20:5(n-3), 22:6(n-3) fatty acids are membrane-bound fatty acids (MbFA), 16:1(n-7), 16:X polyunsaturated fatty acids (PUFA), 18:1(n-7) and 18:X PUFA are of dietary origin (DietFA) (X = 2–4 double bonds), long-chain monounsaturated fatty acids, especially 20:1 and 22:1 fatty acids (both isomers) (MUFA) and saturated fatty acids (SAFA).

Statistical analyses

All data were tested for variance inhomogeneity (Levene test, $P < 0.05$) using STATISTICA 6.1. (Stat. Soft.). Significant differences between group means were analysed using the *T*-test ($P < 0.05$). Datasets involving repeated sampling during the experiment (weight and length) that showed variance inhomogeneity were log-transformed before testing by multiple regression analysis for significant differences between feeding groups.

Prognostic growth models of weight and length gain in both feeding groups were calculated from log-transformed weight and length data for AL and CR groups separately by omitting the start value. The unified growth model included start and end values. The models were tested for variance of inhomogeneity and through regression analysis. Significant differences between separate and unified model were determined using the *F*-test ($P < 0.05$).

Results

Fish growth and response of biochemical parameters to CR and AL feeding

Neither final animal length nor weight differed between AL-fed and CR groups after 8 weeks (55 days) of differential feeding (Table 1). The final liver weight was significantly higher in the AL-fed than in CR fish. The hepatosomatic index (HSI: per cent liver of overall body weight) was three times higher in AL and CR fish compared with the starting value, but without

Table 1 Growth and condition factors in start and feeding groups of young turbot

	Start	<i>Ad libitum</i> fed	Calorie restriction
Body length	11.46 ± 0.66	17.76 ± 1.15*	17.40 ± 1.17*
Body weight	23.06 ± 4.77	113.33 ± 26.12	101.61 ± 24.84
Liver weight	0.146 ± 0.05	2.195 ± 0.68*	1.73 ± 0.77*†
HSI (%)	0.63 ± 0.07	1.94 ± 0.12*	1.70 ± 0.27*
Lipid content (%)	17.7 ± 6.2	21.6 ± 2.8	22.0 ± 3.6

Data are means ± SEM.

*Significant difference between ST and feeding groups.

†Between both feeding groups, $n = 36$, with the exception of lipid content where $n = 8$; significance level: $P < 0.05$.

Table 2 Activities of mitochondrial enzymes citrate synthase (CS) and cytochrome-c oxidase (CCO) in liver and muscle of *ad libitum* and calorie-restricted turbot

	Start	<i>Ad libitum</i> fed	Calorie restriction
<i>Liver</i>			
CS (U g ⁻¹ fwt)	1.91 ± 0.71	1.6 ± 0.22	1.29 ± 0.16†
CCO (U g ⁻¹ fwt)	5.14 ± 1.38	2.09 ± 0.60*	2.60 ± 0.77*
CCO:CS	2.93 ± 1.13	1.33 ± 0.44*	2.06 ± 0.7†
<i>Muscle</i>			
CS (U g ⁻¹ fwt)	0.50 ± 0.14	0.88 ± 0.03*	0.72 ± 0.19*†
CCO (U g ⁻¹ fwt)	0.74 ± 0.11	1.05 ± 0.19*	0.96 ± 0.16*
CCO:CS	1.38 ± 0.35	1.19 ± 0.23	1.20 ± 0.34

Enzyme activities are given as U mg⁻¹ wet weight.

Data are means ± SEM, $n = 8$.

*Significant difference between ST and feeding groups.

†Between feeding groups; significance level: $P < 0.05$.

CCO, cytochrome c oxidase, CS, citrate synthase.

significance between feeding groups, and the liver lipid content (%) remained constant between the start and end of the feeding experiment in both groups (Table 1).

The activities of mitochondrial marker enzymes in young turbot (Table 2) were in the range reported for juvenile cod in white muscle and liver tissue by Couture, Dutij and Guderley (1998), and also comparable to data from adult marine fish (e.g. Yang & Somero 1993). During the feeding experiment, liver CS activity decreased, whereas muscle CS activity increased in both diet groups. Only in liver tissue was CS activity significantly lower in CR than in AL individuals, whereas the activities in muscle did not differ between groups. At the end of the feeding experiment, liver CCO activity per wet weight was significantly lower ($P < 0.05$) than the starting value. In contrast, muscle CCO activity was higher in both feeding

Table 3 Concentrations of reduced (GSH) and oxidized glutathione (GSSG) and glutathione redox ratio (GSH/GSSG) in muscle and liver of *ad libitum* and calorie-restricted turbot

	Start	<i>Ad libitum</i> fed	Calorie restriction
<i>(a) Liver</i>			
GSH _{total}	1281 ± 264	1633 ± 278*	1480 ± 173
GSH	1112 ± 249	1504 ± 252*	1352 ± 157*
GSSG	84.8 ± 13.6	64.5 ± 17.9*	63.8 ± 13.9*
GSH/GSSG	13.2 ± 2.7	24.2 ± 3.8*	21.9 ± 4.7*
<i>(b) Muscle</i>			
GSH _{total}	463 ± 72	525 ± 124	532 ± 27*
GSH	394 ± 61	395 ± 66	460 ± 24*†
GSSG	34.3 ± 9.5	51.6 ± 26.7	36.5 ± 6.7
GSH/GSSG	12.1 ± 2.8	8.4 ± 4.5	13.0 ± 2.7*†

Glutathione concentration in nmol g⁻¹fw as means ± SEM with *n* = 8; ratios are ± SD.

*Significant difference between ST and feeding groups.

†Between feeding groups; significance level: *P* < 0.05.

groups compared with the starting value. At the end of the feeding experiment, CCO activity did not differ between AL-fed and CR fish. The CCO/CS ratios given in Table 2 indicate that the CR fish maintained a similar (muscle) or higher (liver) respiratory capacity per unit of mitochondrial volume as the AL-fed individuals.

The overall content of reduced and oxidized glutathione per milligram tissue weight (GSH_{total} Table 3) was about threefold higher in the liver compared with muscle tissue. During the feeding experiment, GSH_{total} as well as the content of reduced GSH increased significantly in AL group liver, whereas in the CR fish liver the increment of GSH_{total} did not reach significance. Liver GSSG content decreased significantly, and to a similar extent, in both feeding groups so that in both diet groups the liver redox ratio became significantly reduced compared with the ST group without a statistical difference between the CR and AL groups (GSH/GSSG in Table 3a). A minor increase in GSH_{total} was observed in white muscle of both AL and CR group, but reached significance only in the CR group. The concentration of reduced glutathione (GSH) increased in the CR group (*P* < 0.05), whereas the concentration of GSSG in muscle remained unchanged between ST and CR group muscle. The GSSG values in muscle of AL fish were not statistically different because of the high SEM value. However, on calculating the redox ratio for every individual fish muscle, we found AL muscle to be significantly more oxidized than CR fish muscle at the end of the feeding experiment (GSH/GSSG in Table 3b).

Table 4 Oxidative stress indicators in *ad libitum* and calorie-restricted turbot. Concentration of protein carbonyls and thiobarbituric acid reactive lipid-hydroperoxides (TBARS) in liver and muscle tissue

	Start	<i>Ad libitum</i> fed	Calorie restriction
<i>Liver</i>			
Protein carbonyls	1.675 ± 0.802	2.664 ± 0.734*	3.042 ± 1.691
TBARS	1.518 ± 1.025	1.176 ± 0.469	1.322 ± 0.296
<i>Muscle</i>			
Protein carbonyls	6.288 ± 2.637	3.268 ± 1.335*	3.995 ± 0.907*
TBARS	0.149 ± 0.105	0.387 ± 0.153*	0.210 ± 0.079†

The concentrations of protein carbonyls in nmol CO mg⁻¹ protein and for TBARS in μmol g⁻¹fw. Data are means ± SEM, *n* = 8.

*Significant difference between ST and feeding groups.

†Between feeding groups; significance level: *P* < 0.05.

TBARS, thiobarbituric acid reactive lipid hydroperoxides.

Protein oxidation markers (nmol CO mg⁻¹protein) were threefold higher in muscle than liver tissue, whereas liver TBARS (μmol TBARS g⁻¹fw) were 10-fold higher than muscle values in the turbot ST group (Table 4). In the liver, protein carbonylation increased in both the AL group and CR group, but the increase was significant only in the AL group. The TBARS content was slightly diminished in the AL and CR feeding groups relative to the ST values. In muscle tissue of animals from both feeding groups, the protein carbonyl content was significantly reduced to about half the starting value. By contrast, lipid peroxidation in muscle tissue was significantly higher in AL fish than in the ST and in the CR group (Table 4).

Lipid content and fatty acid analysis

Whereas the overall liver lipid content remained constant between start value and both feeding groups (Table 1), differences in fatty acid composition were significant (*P* < 0.05). The typical membrane fatty acids (MbFA) (16:0, 20:5(*n* - 3) and 22:6(*n* - 3)) were more abundant in the ST group liver (55%) as compared with the AL group (45%) and CR group (47%) (Fig. 1). The fatty acids of dietary origin (DietFA) [16:1(*n* - 7), 16:X polyunsaturated fatty acids (PUFA), 18:1(*n* - 7) and 18:X PUFA (X = 2–4 Double bonds)] and long-chain monounsaturated fatty acids, especially 20:1 and 22:1 fatty acids (both isomers) (MUFA), had relatively high proportions in the AL group and

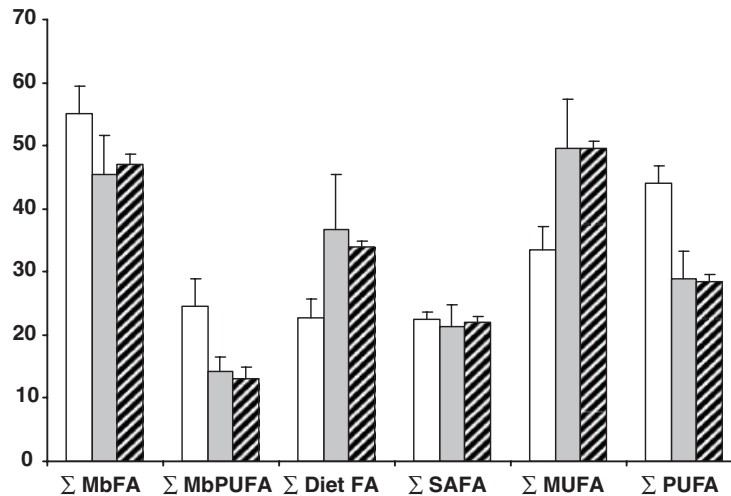


Figure 1 Fatty acid composition (mass %) of liver tissue at the start (ST, white bar, day 36) and after 55 days of *ad libitum* feeding (AL, grey bar) and of 30% calorie-restricted feeding (CR, hatched bar). Sum of membrane fatty acids (MbFA), and polyunsaturated fatty acids of membranes (MbPUFA), dietary fatty acids (Diet FA), saturated fatty acids (SAFA), monounsaturated fatty acids, polyunsaturated fatty acids. (For definition, see also materials and methods part).

CR group with 35% and 49% respectively. Saturated fatty acids (SAFA) appeared in all three groups at almost similar proportions around 22.0%. The proportion of short-chain fatty acids 18:2($n-6$) in liver tissue was reduced in the AL ($6.8\% \pm 0.7$) and in the CR ($6.5\% \pm 1.4$) group, compared with the initial value of $10.6\% \pm 1.4$ (ST group). No significant differences were found between the AL and CR groups. The concentrations of the five major fatty acids in the food (DanEx 1562) itself decreased in the order 22:1($n-11$) (15%), 16:0 (14%), 20:1($n-9$) (12%), 18:1($n-9$) (10%) and 22:6($n-3$), accounting for 7% of total fatty acids.

Discussion

We assumed that a 30% reduction in the food offered would not lead to starvation of the fish and we wished to prove this by comparing growth rate and body maintenance at the level of mitochondrial enzymes, which reflect mitochondrial density (CS) and respiratory capacity (CCO). Also, fatty acid analyses of livers from both turbot feeding groups confirmed that lipid stores derived from dietary lipids to a similar degree in AL and CR fish. At the end of the feeding experiment, the livers contained high proportions of long-chain monounsaturated fatty acids 22:1($n-11$) and 20:1($n-9$), which were also abundant in the commercial feed. In ST group liver, the dominant fatty

acids were 16:0, 20:5($n-3$) and 22:6($n-3$), known to be major phospholipid classes in fish membranes (Sargent 1976). Whereas the individual proportion of these fatty acid compounds varied between the CR and AL groups, their overall amount remained almost constant in all groups.

Although neither the final body length nor the weight in Table 1 differed significantly between the dietary treatments at the end of the experiment (97 days), a prognostic multiple regression growth model for body weight indicated a higher modelled weight gain in AL than in 30%-CR fish (Fig. 2a). The difference in modelled mass gain appeared only after 28 days of calorie restriction, on day 70 of the experimental timeline. The prognostic values and standard errors from both growth models, transformed back to non-logarithmic values, are given in Fig. 2 as weight (a) and length (b) growth models. The trend of a higher body weight gain in the AL group is in line with, but certainly not only due to, the significantly higher liver wet weight in the AL group. The length model (Fig. 2b) provides no indication that AL fish grew faster than CR fish throughout differential feeding.

Deduced from a higher CS activity, AL fish maintained 20% higher mitochondrial density in the liver than CR fish. However, liver aerobic capacity (CCO) did not increase parallel to CS values in AL fish. Thus, while AL feeding may support liver growth and also a larger mitochondrial volume in the organ, weight-specific aerobic capacity and also the aerobic

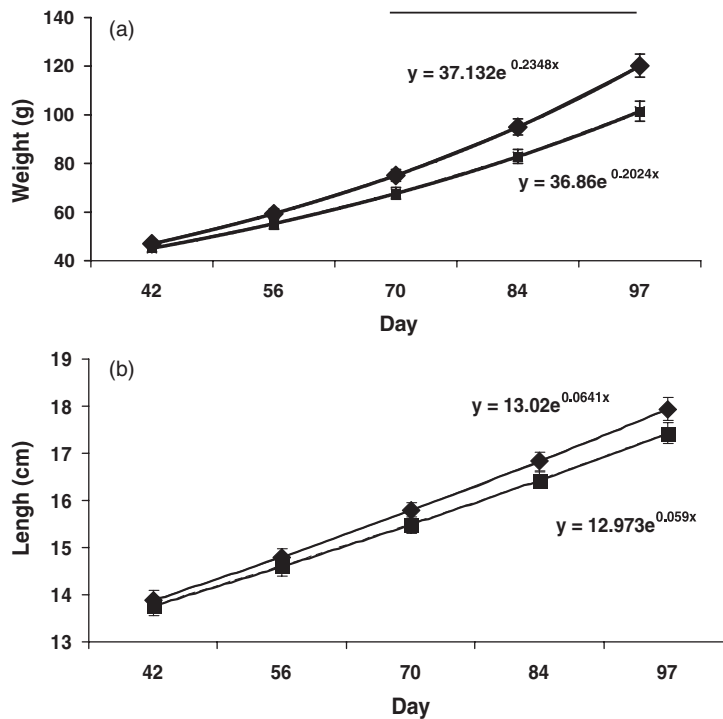


Figure 2 (a) Multiple regression growth model of *ad libitum* feeding (AL, \blacklozenge) and calorie restriction (CR, \blacksquare) turbot body fresh weight. Data as weight means \pm SEM, with $n = 10$ on days 56, 70, 84 and $n = 36$ for days 36, 97 on the experimental timeline. The CR feeding started on day 42. The model indicated significantly lower body weight increment in CR compared with the AL fish from day 70 onwards below the black line. Equations for growth rate are given for each curve. (b) Multiple regression growth model of body length in *ad libitum* feeding (AL, \blacklozenge) and calorie reduced (CR, \blacksquare) turbot feeding groups as forecast values. Data as weight means \pm SEM, with $n = 10$ on days 56, 70, 84 and $n = 36$ for days 36, 97 on the experimental timeline. No significant differences between slopes of both curves indicate calorie restriction to have no effect on animal body length.

capacity per mitochondrion (deduced from CCO:CS in Table 2) appear to be conserved better under CR than under AL conditions in liver tissue. Moreover, the equal respiratory capacity of CR and AL fish muscle is in line with the same swimming activity observed during feeding in both groups (only visual observations). In contrast, cod starved (0% feeding) over 16 weeks showed a 40–59% reduction in CS and CCO (U g^{-1} wet weight) in white muscles (Martínez *et al.* 2003), and scorpionid fish fasted for 100 days lost 35% (*Scorpaena guttata*) and 50% (*Sebastolobus alascanus*) of CS activity when compared with AL-fed controls (Yang & Somero 1993). Interestingly, in the case of the scorpionids, the *in situ* values in freshly caught fish (0.51 U g^{-1} wet mass) were much closer to the fasted ones (0.43 U g^{-1} wet mass) than to AL-fed fish (0.89 U g^{-1} wet mass), indicating the AL diet to be richer than the natural diet and indeed rather overfeeding the fish compared with field con-

ditions. Similar findings were obtained for *in situ* and cultured cod (Martínez *et al.* 2003).

Lower mitochondrial densities in the liver resulting from the 30%-CR condition might affect oxidative stress parameters in two different ways: firstly, in reducing mitochondrial density, CR may limit the production of ROS, ameliorating their damaging effects on tissue lipids and proteins. As a possible drawback, a higher respiratory capacity (CCO activity) per mitochondrion may possibly enhance ROS release from individual mitochondria in the liver, especially under stress when mitochondria release more ROS than under routine conditions (Abele, Philipp, Gonzalez & Puntarulo 2007). Secondly, the 30% feeding reduction could prevent the fish from stabilizing tissue redox rate (total glutathione and glutathione oxidation state) and antioxidant defence (enzyme and vitamin levels), and consequently may cause higher oxidative stress levels.

Our measurements indicate that 30%-CR in young turbot had no effect on total glutathione (GSH_{total}) concentration in either liver or muscle. The glutathione redox ratio in the liver was significantly more reduced in both groups at the end of the feeding experiment compared with the starting value. Also, there was no difference between AL and CR fish, indicating that higher mitochondrial aerobic capacities in liver mitochondria of CR fish have no pro-oxidant effect. Muscle redox ratio and tissue lipids (TBARS) were more oxidized in AL than in CR fish. Therefore, indications of oxidative stress are found in muscle tissue of AL-fed and not in CR turbot.

In a recent study of food deprivation in gilthead seabream, *Sparus aurata* (Pascual *et al.* 2003), fish received very low food offerings from 2% (control), 1, 0.5 down to 0% (full starvation) of animal wet weight over 46 days. The authors found oxidized glutathione (GSSG) and consequently tissue oxidation state to increase from the third week onwards in liver tissue of partially or fully food deprived fish. Tissue oxidation correlated positively with the extent of the food deprivation applied in the different groups. In parallel to the increase in GSSG levels, the lipid peroxidation marker malondialdehyde (MDA) accumulated in the liver of fish subject to reduced feeding. Morales *et al.* (2004) starved common dentex (*Dentex dentex*) over 5 weeks and found that liver glutathione peroxidase increased and liver glutathione reductase decreased following starvation. Although not reported in their study, this is bound to have had a net oxidizing effect on liver glutathione redox ratio. Thus, in contrast to this kind of prolonged strong food deprivation and complete starvation, turbot fed only 30% less than the AL food amount (4% of body weight) were able to stabilize tissue redox state in the liver and appeared even less oxidized in muscle tissue than AL fish.

Morales *et al.* (2004) and Pascual *et al.* (2003) found vastly increased MDA ('TBARS' in our study) levels in fish liver and deduced lipid peroxidation markers to be the most powerful indicator for oxidative stress under food deprivation in fish. In the present study, TBARS content per tissue wet mass decreased from the starting values independently of whether fish were fed an AL or a CR diet (Table 4), reflecting the absence of oxidative injury in both groups. As in our turbot study, TBARS (MDA) in seabream muscles was below $0.5 \mu\text{mol g}^{-1} \text{fw t g}$ under all feeding conditions (Pascual *et al.* 2003). Protein oxidation levels were not affected by CR, indicating protein turnover and also antioxidant defence in the protein fraction of

young turbot tissue to be equally protective in AL and CR fish.

Differences between CR and starvation in fish

In spite of differences in experimental design and in the parameters analysed in published papers, some significant differences emerge that distinguish responses to starvation and calorie restriction in fish. Whereas starvation for 5 weeks led to a significant loss of body weight in *D. dentex* (Morales *et al.* 2004), 8 weeks (55 days) of calorie restriction in young turbot did not lead to mass loss and only the calculated weight increment was lower in the CR group at the end of the experiment. Starvation reduced mitochondrial enzyme activities in white muscle of starved cod (Martínez *et al.* 2003), whereas in 30%-CR turbot white muscle aerobic capacity remained constant. In dentex liver, starvation led to increased CS activity. In contrast, CR turbot CS activity was even reduced under calorie restriction in the liver.

Whereas our fish received 4% of body weight daily in the AL group (two times 2%) and a 30% reduction from this in the CR group over 8 weeks with no apparent effect on the HSI, in gilthead seabream the HSI remained unaffected as long as the animals received 2% of their body weight daily in a similar time span (46 days). However, HSI in seabream decreased when food offering was lowered to 1% of body weight or less per day or during complete starvation (Pascual *et al.* 2003).

Finally, liver glutathione redox ratio that tends to be more oxidized in starved fish is kept constant under CR. Neither lipid peroxidation markers per gram wet weight nor protein carbonyls accumulated under CR in young turbot, whereas MDA (TBARS) increased significantly under severe food deprivation or during complete starvation (Pascual *et al.* 2003; Morales *et al.* 2004). Although not investigated in our study, it appears likely that antioxidant enzyme activities are maintained constant during calorie restriction in turbot, whereas they can even be induced during starvation (Guderley *et al.* 2003; Morales *et al.* 2004).

In conclusion, we found that 30% calorie restriction in juvenile turbot had no adverse effects during 8 weeks. Production was not significantly diminished, and no negative effect was observed on the fitness and aerobic capacity of the fish or on oxidative stress parameters. Thus, calorie restriction may be included as a strategy in the feeding regime of fish that

are to be transported (e.g. juvenile turbot to their grow-out site), to reduce excretion within the limited space of transport tanks. Longer experiments applying CR diets are needed to see if calorie restriction can also be applied as a more general strategy to ease feeding management without a major cutback on weight gain, and to demonstrate whether fish indeed remain healthier in aquaculture under reduced feeding.

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