JOURNAL OF EXPERIMENTAL ZOOLOGY 303A:761–775 (2005)

# Interactive Effects of High Stocking Density and Food Deprivation on Carbohydrate Metabolism in Several Tissues of Gilthead Sea Bream *Sparus auratus*

SUSANA SANGIAO-ALVARELLOS[1], JOSÉ M. GUZMÁN[2], RAÚL LÁIZ-CARRIÓN[2], JESÚS M. MÍGUEZ[1], MARÍA P. MARTÍN DEL RÍO[2], JUAN M. MANCERA[2], AND JOSÉ L. SOENGAS[1]*
[1]*Laboratorio de Fisioloxía Animal, Facultade de Ciencias do Mar, Universidade de Vigo, 36310 Vigo, Spain*
[2]*Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain*

*ABSTRACT* The influence of high stocking density (HSD) and food deprivation was assessed on carbohydrate metabolism of several tissues of gilthead sea bream *Sparus auratus* for 14 days. Fish were randomly assigned to one of four treatments: (1) fed fish under normal stocking density (NSD) ($4\,kg\,m^{-3}$); (2) fed fish under HSD ($70\,kg\,m^{-3}$); (3) food-deprived fish under NSD; and (4) food-deprived fish under HSD. After 14 days, samples were taken from the plasma, liver, gills, kidney and brain for the assessment of plasma cortisol, levels of metabolites and the activity of several enzymes involved in carbohydrate metabolism. HSD conditions alone elicited important changes in energy metabolism of several tissues that in some cases were confirmatory (5-fold increase in plama cortisol, 20% increase in plasma glucose, 60% decrease in liver glycogen and 20% increase in gluconeogenic potential in the liver) whereas in others provided new information regarding metabolic adjustments to cope with HSD in the liver (100% increase in glucose phosphorylating capacity), gills (30% decrease in capacity for phosphorylating glucose), kidney (80% increase in the capacity of phosphorylating glucose) and brain (2.5-fold increase in ATP levels). On the other hand, food deprivation alone resulted in increased plasma cortisol, and metabolic changes in the liver (enhanced gluconeogenic and glycogenolytic potential of 13% and 18%, respectively) and brain (10% increase in glycolytic capacity), confirmatory of previous studies, whereas new information regarding metabolic adjustments during food deprivation was obtained in the gills and kidney (decreased lactate levels in both tissues of 45% and 55%, respectively). Furthermore, the results obtained provided, for the first time in fish, information indicating that food deprivation increased the sensitivity of gilthead sea bream to the stress induced by HSD compared with the fed controls, as demonstrated by increased plasma cortisol levels (50% increase vs. fed fish) and a further increase in the capacity to export glucose mobilized from liver glycogen stores (70% decrease vs. fed fish). These results lend support for a cumulative effect of both stressors on plasma cortisol and parameters assessed on carbohydrate metabolism in the present experiments, and provide information regarding reallocation of metabolic energy to cope with simultaneous stressors in fish. *J. Exp. Zool. 303A:761–775, 2005.* 

In the aquaculture environment, the exposure to stressors is commonplace due to regular management procedures (weighing, transporting, etc.) or for economic reasons that increased rearing densities (Barton and Iwama, '91; Ruane et al., 2002). Not surprisingly, most studies on the physiology of stress in fish have been directed towards the effects of common stressors related to handling procedures in laboratory work and

Grant sponsor: Ministerio de Ciencia y Tecnología, Spain and FEDER; Grant number: BOS2001-4031-C02-01 and BOS2001-4031-C02-02 and VEM2003-20062; Grant sponsor: Xunta de Galicia, Spain; Grant number: PGIDT01PXI30113PR|PGIDT04PXIC31208PN; Grant sponsor: Universidade de Vigo; Grant number: c46164102.
*Correspondence to: Dr. José L. Soengas, Laboratorio de Fisioloxía Animal, Facultade de Ciencias do Mar, Edificio de Ciencias Experimentais, Universidade de Vigo, E-36310 Vigo, Spain.
E-mail: jsoengas@uvigo.es
Received 21 September 2004; Accepted 23 May 2005
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.a.203.

aquaculture. Responses to stress-related disturbances in fish are often characterized as primary, secondary or tertiary. Such a stressor elicits a primary stress response; this includes elevations in plasma cortisol and catecholamine levels (Barton and Iwama, '91; Wendelaar Bonga, '97). These hormonal responses initiate a series of secondary stress responses, including elevation in glycogen and/or glucose metabolism, which enable fish to avoid or cope with the maladaptive effects of the stressor. Thus, a central aspect of stress adaptation is the reallocation of metabolic energy away from investment activities (i.e., growth and reproduction) and towards activities that require intensification to restore homeostasis, such as respiration, locomotion, hydromineral regulation and tissue repair (Wendelaar Bonga, '97). In this way, stress may increase the importance of carbohydrate metabolism in the whole animal energy budget, and, not surprisingly, blood glucose levels generally increase during stress.

Fish held at high stocking densities (HSD) are generally considered to be exposed to chronic-stressor situations that predispose the fish to infection, reproductive impairment, etc. and impose severe energy demands (Vijayan et al., '90; Scott-Thomas et al., '92; Rotllant et al., 2000, 2001). The extra energy requirements are perhaps met by mobilizing body resources, resulting in lower growth and performance. Only a few studies have explored the metabolic changes occurring in the liver associated with HSD in teleosts (Vijayan et al., '90; Scott-Thomas et al., '92; Rotllant et al., 2000, 2001). However, no studies have been performed assessing metabolic changes induced by HSD in tissues other than the liver.

Food deprivation is also a common type of stress that fish experience in both laboratory and field experiments. The maintenance of glycemia during food deprivation is directly related to the capacity of mobilization of hepatic glycogen, at least during the initial stages of fasting, and also depends on the subsequent activation of hepatic gluconeogenesis and reduction in the rate of glucose utilization (Sheridan and Mommsen, '91; Navarro and Gutiérrez, '95). The information regarding metabolic changes in tissues other than the liver under food deprivation conditions is also limited to some studies performed in the brain (Soengas et al., '96, '98; Figueroa et al., 2000; Tripathi and Verma, 2003), muscle (Collins and Anderson, '97; Kirchner et al., 2005), kidney and intestine (Kirchner et al., 2005).

Considerable work has been done on the effects of acute, simple stressors and chronic stress on the energy metabolism of fish (Barton and Iwama, '91; Wendelaar Bonga, '97). However, studies that evaluate the metabolic capacities of fish subjected to combined stressors are limited in spite of the fact that multiple stressors are the norm under field conditions (Rotllant and Tort, '97; Wagner et al., '97).

Since the nutritional state has a profound influence on both the stress response and cortisol-induced changes in metabolism, any alteration in feeding could modify the stress response and the resulting metabolic adaptation of the fish to an additional stressor (Vijayan and Moon, '92; Vijayan et al., '96). In this regard, only limited information is available regarding effects of food deprivation after handling (Vijayan and Moon, '92; Reubush and Heath, '96) or toxicant exposure (Jorgensen et al., 2002) on the energy metabolism of fish liver. As far as we are aware, no studies have dealt with the simultaneous effects of HSD and food deprivation on energy metabolism in different tissues of any teleost species.

Most studies on metabolic adjustments to stress in fish have been limited to salmonids and very little information is available for other fish species (Vijayan et al., '97). Gilthead sea bream (*Sparus auratus*) is a widely cultured fish in Southern Europe and is subjected to routine management stress including HSD and food deprivation (Arends et al., '99, 2000; Montero et al., '99, 2001; Rotllant et al., 2000, 2001). This species responds to stress with the stimulation of the hypothalamus–pituitary–interrenal axis and the subsequent elevation in plasma cortisol concentration (Arends et al., '99, 2000; Rotllant et al., 2000, 2001). However, little information is available regarding metabolic adjustments to stress in this species (Montero et al., '99, 2001).

Therefore, the objective of the present study was to examine, in gilthead sea bream, the effect of 14 days under HSD conditions on plasma cortisol, and metabolite levels as well as the activities of several enzymes involved in the carbohydrate metabolism of different tissues, and to assess whether or not food deprivation modifies the stress response of carbohydrate metabolism elicited by HSD conditions.

## MATERIALS AND METHODS

### *Fish*

Immature male gilthead seabream (*S. auratus* L., 400–450 g body weight) were provided by

Planta de Cultivos Marinos (CASEM, Universidad de Cadiz, Puerto Real, Cádiz, Spain) and transferred to the laboratories at the Faculty of Marine Science (Puerto Real, Cádiz). They were acclimated to SW in 300 l aquaria for at least 2 weeks in flow-through tanks providing a constant supply of fresh seawater (38 ppt salinity, 1,000 mOsm/kg $H_2O$), before the experiments. During the experiments, fish were maintained under natural photoperiod (April) and constant temperature (18°C) during which the common water quality criteria (hardness, pH, levels of oxygen, carbon dioxide, hydrogen sulfide, nitrite, nitrate, ammonia, calcium, chlorine and suspended solids) were assessed, with no major changes being observed. Fish were fed once daily, to satiety, with commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain. Proximate food analysis was 48% crude protein, 6% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MegaJ/kg of feed). They were fasted for 24 hr before sampling. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and the University of Cádiz (Spain) for the use of laboratory animals.

### *Experimental design*

Fish were randomly assigned to 300 l experimental tanks containing a plastified iron wire-net cage with a total volume of 250 l (inner diameter of cage 60 cm) to obtain a fish density of 4 kg $m^{-3}$. The fish were allowed to acclimate to the experimental tank for 7 days. The wire-net cage in the tank was lifted (water depth about 15 cm) to increase the stocking density from 4 to 70 kg $m^{-3}$. Each tank was randomly assigned to one of four treatments (two replicates/treatment): (1) fed fish under normal stocking density (NSD, 4 kg $m^{-3}$); (2) fed fish under HSD (70 kg $m^{-3}$); (3) food-deprived fish under NSD; and (4) food-deprived fish under HSD. The fish that fed were allowed to eat to satiety once daily with pelleted diets, whereas the food-deprived fish were deprived of food from the start of the experiment onwards. After 14 days, 12 fish from each treatment (six from each tank) were removed by dip-net and tissue samples were taken as described below. The period of food deprivation was chosen based on other studies showing significant changes in metabolic status after 2 weeks of food deprivation (see Navarro and Gutiérrez, '95).

### *Sampling*

Fish were anesthetized with 2-phenoxyethanol (0.1% v/v) and weighed. Blood (approx. 2 ml) was obtained in ammonium-heparinized sterile syringes from the caudal peduncle. Plasma samples were obtained after centrifugation of blood (30 sec at 13,000*g*; Eppendorf 5415R), deproteinized immediately (using 6% perchloric acid) and neutralized (using 1 mol $l^{-1}$ potassium bicarbonate) before freezing on liquid nitrogen and storage at −80°C until further assay. The brain, kidney, liver and gills were rinsed with saline, weighed, freeze-clamped in liquid nitrogen and stored at −80°C until assayed (in less than 4 weeks).

### *Analytical techniques*

#### Plasma cortisol

Cortisol levels were measured (in duplicate) in plasma by indirect enzyme immunoassay (EIA) validated for gilthead sea bream and other fish species (Tintos et al., submitted). Briefly, Covalink microplates (Nunc) pretreated with disuccinimidyl suberate were coated with a given amount of a conjugate of bovine serum albumin (BSA) with the active ester of 3-carboxymethyl oxime prepared with cortisol. After incubation and blocking with BSA, competition was started by addition of samples and anti-cortisol antibody raised in rabbit. Goat anti-rabbit IgG conjugated peroxidase was added as second antibody and then incubated with OPD as substrate. Reaction was stopped with 0.1 M HCl and absorbance was read at 450 nm in an automatic plate reader. The standard curve was linear (logit/log) from the lower limit of sensitivity of the assay (0.3 ng/ml) to approximately 3,000 ng/ml. Dose–response inhibition curves using serially diluted plasma samples consistently showed parallelism with the standard curve using cortisol. The EIA satisfied the strictest criteria of specificity (testing cross-reactivity with other steroids), reproducibility (interassay coefficient of variation $<6\%$), precision (intra-assay coefficient of variation $<4\%$) and accuracy (average recovery $>98\%$).

#### Plasma metabolites

Plasma glucose and lactate were measured spectrophotometrically, using commercial kits from Spinreact (Barcelona, Spain) adapted to microplates.

## Tissue metabolites

Frozen brain, kidney, liver and gill samples were minced, on a chilled Petri dish, to very small pieces that were mixed and while still frozen, divided into two homogeneous aliquots to assess enzyme activities and metabolite levels, respectively. The tissue used for the assessment of metabolite levels was homogenized immediately by ultrasonic disruption in the cold with 7.5 vols of ice-cooled 6% perchloric acid and neutralized (using 1 mol $l^{-1}$ potassium bicarbonate). The homogenate was centrifuged (2 min at 13,000*g*, Eppendorf 5415R), and the supernatant used for assays. Tissue lactate and ATP levels were determined enzymatically in duplicates using commercial kits (Spinreact and Sigma Chemical (St. Louis, MO) for lactate and ATP, respectively). Tissue glycogen levels were assessed in duplicates using the Keppler and Decker method ('74). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined enzymatically using a commercial kit (Biomérieux, Barcelona, Spain).

## Tissue enzyme activities

The tissue used for the assessment of enzyme activities was homogenized by ultrasonic disruption in the cold with 10 vols of ice-cold stopping buffer containing 50 mmol $l^{-1}$ imidazole-HCl (pH 7.5), 1 mmol $l^{-1}$ 2-mercaptoethanol, 50 mmol $l^{-1}$ NaF, 4 mmol $l^{-1}$ EDTA, 250 mmol $l^{-1}$ sucrose and 0.5 mmol $l^{-1}$ *p*-methyl-sulfonyl-fluoride (added as dry crystals immediately before homogenization). The homogenate was centrifuged (2 min at 13,000*g*, Eppendorf 5415R) and the supernatant used for assays. In those cases where non-cytosolic enzymes were assessed, appropriate centrifugations were carried out to obtain samples.

The activities of several enzymes representative of the major pathways of carbohydrate metabolism (glycogen phosphorylase (GPase), pyruvate kinase (PK), 6-phosphofructo 1-kinase (PFK), hexokinase (HK), glucose 6-phosphatase (G6Pase), glucose 6-phosphate dehydrogenase (G6PDH)) were determined using a Unicam UV6-220 spectrophotometer (Thermo Unicam, Waltham, MA). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (0.05 ml), at a pre-established protein concentration, omitting the substrate in control cuvettes (final volume 1.35 ml), and allowing the reactions to proceed at 15°C for pre-established times. No changes were found in tissue protein levels in any of the groups studied, and therefore enzyme activities are expressed in terms of $mg^{-1}$ protein. Homogenate protein was assayed in triplicate as detailed by Bradford ('76), using BSA (Sigma, USA) as standard. Enzyme analyses were all carried out to achieve maximum rates in each tissue, as defined in preliminary tests. The specific conditions for enzyme assays were described previously (Laiz-Carrión et al., 2002, 2003; Sangiao-Alvarellos et al., 2003a, b, 2004, 2005), after adaptation of methods described for salmonids (Soengas et al., '96, '98).

### *Statistics*

The effect of SD conditions (normal and high) and feeding conditions (fed and food deprived) as well as its possible interaction in parameters assessed was analyzed using a two-way ANOVA with SD conditions and feeding conditions as main factors. When significant differences were obtained from the ANOVA, multiple comparisons were carried out using the Student–Newman–Keuls test. Significance level was set at $P<0.05$.

## RESULTS

No mortality, health disturbances or any alterations in behavior were observed in any group of fish throughout the study. Fed fish under NSD conditions gained an average ($\pm$SEM) of $28\pm0.4$ g in weight after 14 days. In contrast, fish in the remaining groups lost weight after 14 days (average lost values of $12\pm0.2$, $2\pm0.3$, and $17\pm2.1$ g for food-deprived fish in NSD, fed fish in HSD and food-deprived fish in HSD, respectively). *P*-values resulting from the two-way ANOVA of all parameters assessed are displayed in Table 1.

The parameters assessed in plasma are described in Fig. 1. Plasma cortisol levels increased significantly (3–5-fold) in HSD compared with NSD groups and food-deprived fish also displayed higher levels (1.5–2-fold) than fed fish. Plasma glucose levels increased significantly (approximately 20%) in HSD compared with NSD groups and food-deprived fish under HSD conditions displayed higher levels (15% increase) than those of fed fish under the same stocking density condition. Plasma lactate levels decreased in HSD groups when compared with NSD fish (35–40%) and food-deprived fish under NSD conditions displayed a 1.5-fold decrease compared

*TABLE 1. P-Values from two-way ANOVA of parameters measured in plasma, liver, gills, kidney, and brain of gilthead seabream after 14 days of maintenance at high stocking density and/or food deprivation. Stocking density (normal, 4 kg.m-3; high, 70 kg.m-3) and feeding conditions (fed and food deprived) are the main factors. ns, no significant**

| Tissue | Parameter | Density | Feeding | Density × feeding |
|---|---|---|---|---|
| Plasma | Cortisol levels | <0.001 | <0.001 | <0.001 |
| | Glucose levels | <0.001 | <0.001 | ns |
| | Lactate levels | <0.001 | <0.001 | <0.001 |
| Liver | Glycogen levels | <0.001 | <0.001 | <0.001 |
| | Lactate levels | 0.007 | ns | ns |
| | ATP levels | <0.001 | <0.001 | ns |
| | GPase activity (total) | 0.003 | <0.001 | 0.005 |
| | GPase activity (% $a$) | 0.01 | <0.001 | ns |
| | PK activity (Optimal) | 0.041 | ns | ns |
| | PK activity (activity ratio) | ns | ns | ns |
| | PK activity (activation ratio) | ns | ns | ns |
| | FBPase activity | ns | ns | ns |
| | G6Pase activity | <0.001 | ns | ns |
| | G6PDH activity | ns | ns | 0.042 |
| Gills | Glycogen levels | ns | 0.063 | 0.011 |
| | Lactate levels | 0.011 | 0.022 | <0.001 |
| | GPase activity (total) | 0.038 | ns | ns |
| | GPase activity (% $a$) | 0.048 | ns | ns |
| | PK activity (Optimal) | 0.008 | ns | ns |
| | PK activity (activity ratio) | ns | ns | ns |
| | PK activity (activation ratio) | ns | ns | ns |
| | HK activity | ns | ns | ns |
| | G6PDH activity | 0.006 | ns | ns |
| Kidney | Glycogen levels | 0.036 | ns | ns |
| | Lactate levels | <0.001 | <0.001 | <0.001 |
| | GPase activity (total) | ns | ns | ns |
| | GPase activity (% $a$) | ns | ns | ns |
| | PK activity (Optimal) | <0.001 | ns | ns |
| | PK activity (activity ratio) | ns | ns | ns |
| | PK activity (activation ratio) | ns | ns | ns |
| | HK activity | <0.001 | ns | ns |
| Brain | Glycogen levels | ns | ns | ns |
| | Lactate levels | 0.048 | 0.009 | ns |
| | ATP levels | 0.018 | ns | ns |
| | GPase activity (total) | 0.031 | ns | ns |
| | GPase activity (% $a$) | ns | ns | ns |
| | PFK activity (Optimal) | ns | 0.035 | ns |
| | PFK activity (activity ratio) | ns | 0.01 | ns |
| | PFK activity (activation ratio) | ns | ns | ns |
| | HK activity | ns | ns | ns |
| | G6PDH activity | ns | ns | ns |

*Stocking density (normal, 4 kg m$^{-3}$; high, 70 kg m$^{-3}$) and feeding conditions (fed and food deprived) are the main factors; ns, not significant.

with fed fish under the same stocking density condition.

Liver glycogen levels (Fig. 2) decreased 2.5–4 fold in food-deprived groups compared with fed groups and levels were also lower (60–200% decrease) in HSD than in NSD groups. Lactate levels in the liver (Fig. 2) were lower in food-deprived fish than in fed fish only under NSD conditions (34% decrease) and were also lower in HSD than in NSD fish but only under fed conditions (64% decrease). ATP levels were lower (approximately 65%) in food-deprived groups than

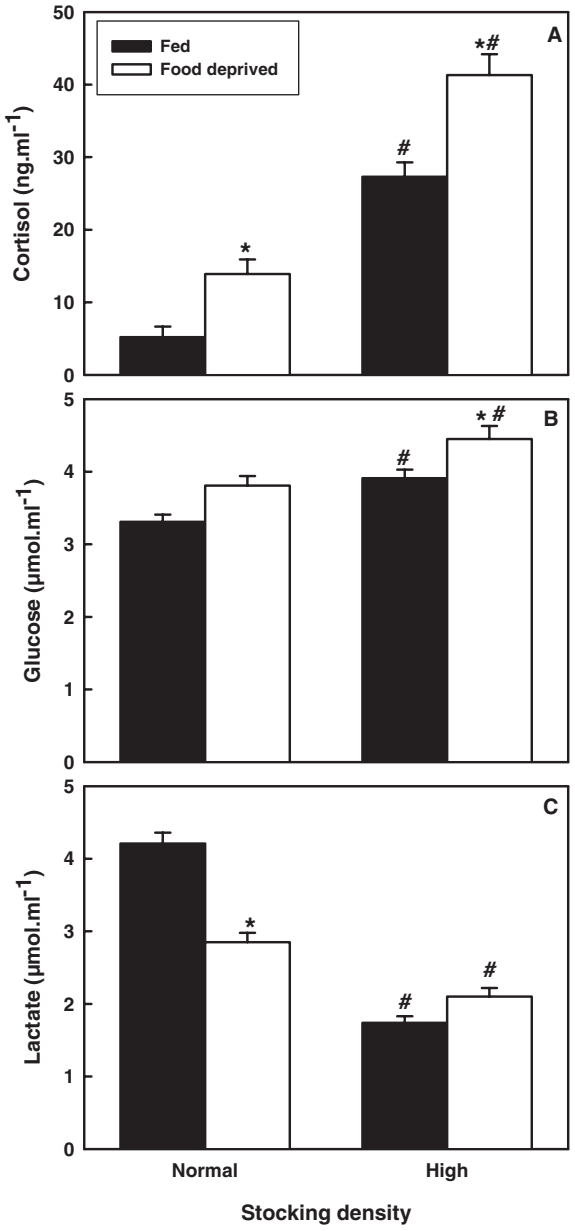


Fig. 1. Effects of high stocking density and/or food deprivation for 14 days on levels of cortisol (**A**), glucose (**B**) and lactate (**C**) in plasma of the gilthead sea bream. Normal stocking density was $4\,kg\,m^{-3}$, high stocking density was $70\,kg\,m^{-3}$. Data represent mean $\pm$ SEM of 12 measurements. *, significantly different ($P<0.05$) from fed group at the same density. $^{\#}$, significantly different ($P<0.05$) from normal density at the same feeding condition.

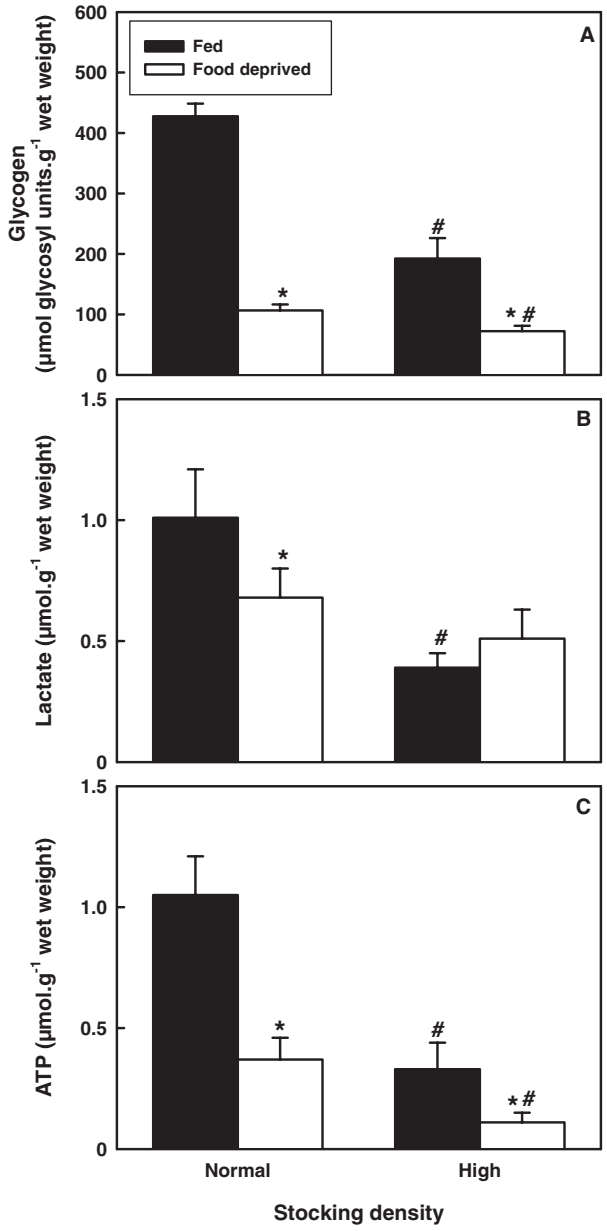


Fig. 2. Effects of high stocking density and/or food deprivation for 14 days on levels of glycogen (**A**), lactate (**B**) and ATP (**C**) in the liver of the gilthead sea bream. Further details as in legend to Fig. 1.

in fed groups and further decreased (approximately 70%) in HSD groups compared with NSD groups.

Liver enzyme activities are displayed in Table 2. GPase total activity was lower (85% decrease) in food-deprived than in fed fish under NSD conditions and under fed conditions, activity was lower in HSD than in NSD fish (65% decrease). The percentage GPase *a* was higher (26–76% increase) in food-deprived groups than in fed groups and also higher in HSD than in NSD, but only under fed conditions (55% increase). As for PK activity, only the optimal activity of the enzyme displayed any significant differences; particularly, values in

HSD groups were lower than those in NSD fish (approximately 15%). FBPase activity was higher (20% increase) in HSD than in NSD fish only under fed conditions, and also higher in food-deprived fish than in fed fish under NSD conditions (13% increase). G6Pase activity was higher (100% increase) in HSD groups than in NSD groups and in NSD, fish activity was higher (45% increase) in food-deprived fish than in fed fish. Finally, G6PDH activity was lower in HSD than in NSD fish under fed conditions (37% decrease) and activity was also lower in fed fish than in food-deprived fish under HSD conditions (60% decrease).

Glycogen levels in gills (Fig. 3) were lower (40% decrease) in food-deprived fish than in fed fish under HSD conditions and HSD fish displayed lower levels (34% decrease) than those of NSD fish under food deprivation conditions. Lactate levels in gills (Fig. 3) were lower (44% decrease) in food-deprived than in fed fish under NSD conditions whereas levels were lower (40% decrease) in HSD than in NSD fish under fed conditions.

Enzyme activities in gills are shown in Table 3. As for GPase activity, total activity was lower (15–35% decrease) in HSD groups than in NSD groups. The % GPase *a* was higher (15% increase) in HSD than in NSD food-deprived fish, and a 5-fold increase was also observed when comparing food-deprived vs. fed fish under HSD conditions. As for PK activity, only the optimal activity displayed changes where values were lower (approximately 35%) in HSD groups than in NSD groups. HK activity of HSD fish was lower (30% decrease) than that of NSD under fed conditions. Finally, G6PDH activity was lower (18–37%) in HSD groups than in NSD groups.

*TABLE 2. Effects of high stocking density and/or food-deprivation for 14 days on activities of glycogen phosphorylase (GPase), pyruvate kinase (PK), fructose 1,6-bisphosphatase (FBPase), glucose 6-phosphatase (G6Pase), and glucose 6-phosphate dehydrogenase (G6PDH) in liver of gilthead sea bream. Normal stocking density was 4 kg.m$^{-3}$, high stocking density was 70 kg.m$^{-3}$. Data represent mean ± SEM of 12 measurements. *, significantly different ($P < 0.05$) from fed group at the same density. #, significantly different ($P < 0.05$) from normal density at the same feeding condition**

| Parameter | Density | Feeding conditions: Fed | Feeding conditions: Food deprived |
|---|---|---|---|
| GPase activity | | | |
| Total activity | Normal | 0.63 ± 0.11 | 0.11 ± 0.02* |
| (U mg$^{-1}$ protein) | High | 0.22 ± 0.04# | 0.10 ± 0.006 |
| % GPase *a* | Normal | 34.2 ± 3.02 | 60.7 ± 5.02* |
| | High | 53.1 ± 4.93# | 67.1 ± 6.61* |
| PK activity | | | |
| Optimal activity | Normal | 0.61 ± 0.04 | 0.65 ± 0.05 |
| (U mg$^{-1}$ protein) | High | 0.53 ± 0.03# | 0.55 ± 0.02# |
| Activity ratio | Normal | 0.33 ± 0.14 | 0.10 ± 0.02 |
| | High | 0.08 ± 0.04 | 0.11 ± 0.06 |
| Fructose 1,6-$P_2$ | Normal | 0.13 ± 0.03 | 0.07 ± 0.01 |
| activation ratio | High | 0.04 ± 0.02 | 0.07 ± 0.03 |
| FBPase activity | | | |
| Optimal activity | Normal | 0.59 ± 0.02 | 0.67 ± 0.02* |
| (U mg$^{-1}$ protein) | High | 0.71 ± 0.04# | 0.70 ± 0.05 |
| G6Pase activity | | | |
| Optimal activity | Normal | 1.44 ± 0.10 | 2.09 ± 0.09* |
| (U mg$^{-1}$ protein) | High | 3.01 ± 0.41# | 3.16 ± 0.19# |
| G6PDH activity | | | |
| Optimal activity | Normal | 2.71 ± 0.14 | 2.61 ± 0.29 |
| (U mg$^{-1}$ protein) | High | 1.99 ± 0.17# | 2.76 ± 0.23* |

*Normal stocking density was 4 kg m$^{-3}$, high stocking density was 70 kg m$^{-3}$. Data represent mean ± SEM of 12 measurements. *, significantly different ($P < 0.05$) from fed group at the same density. #, significantly different ($P < 0.05$) from normal density at the same feeding condition.

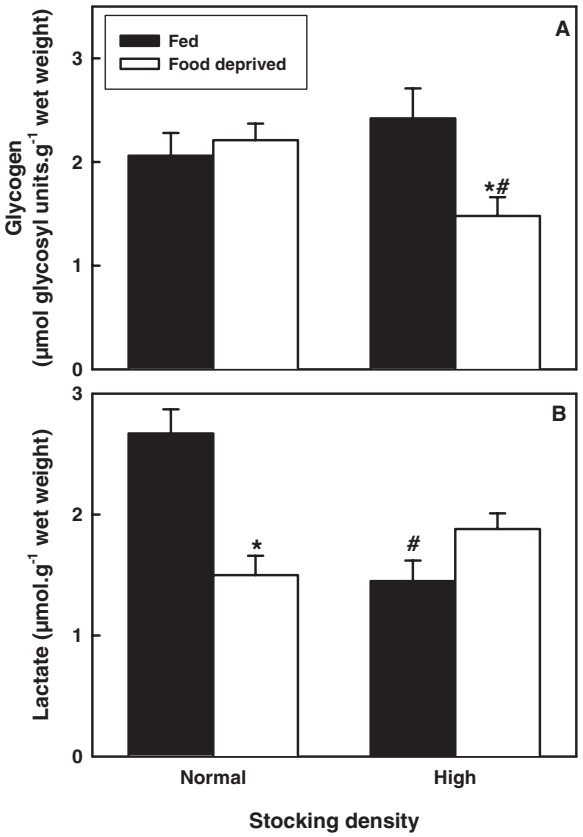


Fig. 3. Effects of high stocking density and/or food deprivation for 14 days on levels of glycogen (**A**) and lactate (**B**) in gills of the gilthead sea bream. Further details as in legend to Fig. 1.

*TABLE 3. Effects of high stocking density and/or food-deprivation for 14 days on activities of glycogen phosphorylase (GPase), pyruvate kinase (PK), hexokinase (HK), and glucose 6-phosphate dehydrogenase (G6PDH) in gills of gilthead sea bream. Further details as in legend to Table 2**

| Parameter | Density | Feeding conditions: Fed | Feeding conditions: Food deprived |
|---|---|---|---|
| GPase activity | | | |
| Total activity | Normal | 0.12 ± 0.007 | 0.14 ± 0.03 |
| ($U\,mg^{-1}$ protein) | High | 0.10 ± 0.009$^{\#}$ | 0.08 ± 0.02$^{\#}$ |
| % GPase *a* | Normal | 2.70 ± 1.19 | 3.96 ± 1.89 |
| | High | 1.82 ± 1.05$^{\#}$ | 6.05 ± 0.01*$^{\#}$ |
| PK activity | | | |
| Optimal activity | Normal | 6.42 ± 0.33 | 5.63 ± 0.48 |
| ($U\,mg^{-1}$ protein) | High | 4.99 ± 0.31$^{\#}$ | 4.48 ± 0.63$^{\#}$ |
| Activity ratio | Normal | 0.60 ± 0.02 | 0.59 ± 0.01 |
| | High | 0.61 ± 0.01 | 0.62 ± 0.01 |
| Fructose 1,6-$P_2$ | Normal | 0.99 ± 0.02 | 0.98 ± 0.02 |
| activation ratio | High | 0.98 ± 0.02 | 0.99 ± 0.01 |
| HK activity | | | |
| Optimal activity | Normal | 0.87 ± 0.08 | 0.67 ± 0.06 |
| ($U\,mg^{-1}$ protein) | High | 0.62 ± 0.08$^{\#}$ | 0.66 ± 0.07 |
| G6PDH activity | | | |
| Optimal activity | Normal | 1.43 ± 0.10 | 1.30 ± 0.14 |
| ($U\,mg^{-1}$ protein) | High | 1.05 ± 0.06$^{\#}$ | 1.07 ± 0.11$^{\#}$ |

*Further details as in legend to Table 2.

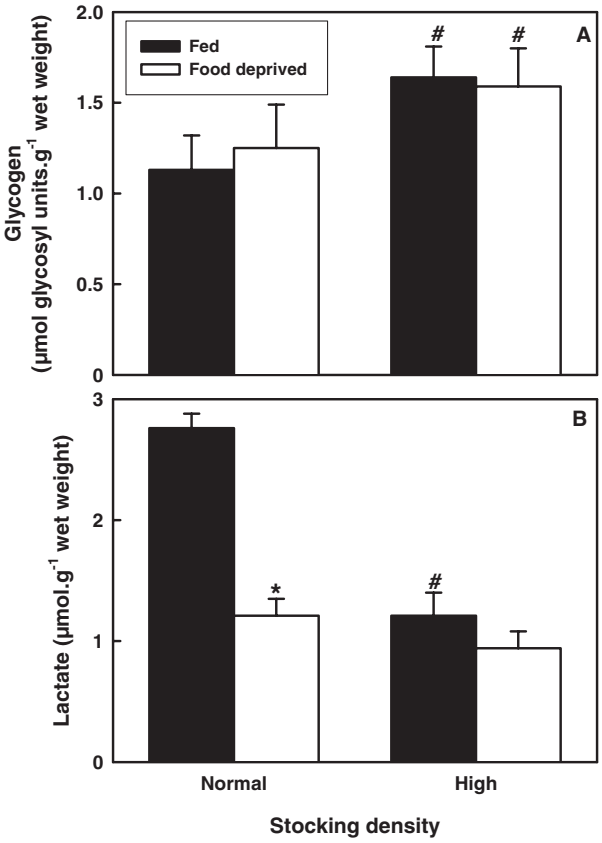


Fig. 4. Effects of high stocking density and/or food deprivation for 14 days on levels of glycogen (**A**) and lactate (**B**) in the kidney of the gilthead sea bream. Further details as in legend to Fig. 1.

Glycogen levels in kidney (Fig. 4) were higher (27–45% increase) in HSD groups than in NSD groups. Lactate levels in kidney (Fig. 4) decreased (55%) in food-deprived fish compared with fed fish under NSD conditions and levels also decreased (50%) in HSD fish than in NSD fish under fed conditions.

Enzyme activities in kidney are displayed in Table 4. As for PK activity, only the optimal activity displayed changes where values were higher (28–52% increase) in HSD groups than in NSD groups. HK activity was higher (40–80% increase) in HSD groups than in NSD groups.

Brain glycogen levels (Fig. 5) were not significantly different between groups. Brain lactate levels (Fig. 5) decreased (20–30%) in HSD groups compared with NSD groups. Brain ATP levels (Fig. 5) were higher (250–400% increase) in HSD than in NSD groups.

Finally, brain enzyme activities are displayed in Table 5. Total GPase activity was higher (10–18% increase) in HSD than in NSD fish. The optimal activity of PFK as well as the activity ratio of the enzyme were higher (10%) in all food-deprived groups than in their respective fed groups.

## DISCUSSION

### *Effects of HSD*

The rearing of fish under crowded conditions such as stocking densities has been shown to negatively affect growth, influence metabolite levels and modulate immune functions (Barton and Iwama, '91; Ruane et al., 2002). Accordingly, growth was also affected by stocking density conditions in the present study.

Only a few studies have attempted to estimate the extent of the additional energy requirements associated with increased density (Scott-Thomas et al., '92; LeFrançois et al., 2001; Ruane et al., 2002). These studies generally address the fact that fish under HSD conditions showed a lowering of aerobic processes and enhancement of liver gluconeogenesis and little carbohydrate utilization (Scott-Thomas et al., '92).

In the present study, HSD conditions elicited a 2.5-fold decrease in glycogen levels in the liver,

*TABLE 4. Effects of high stocking density and/or food-deprivation for 14 days on activities of glycogen phosphorylase (GPase), pyruvate kinase (PK), and hexokinase (HK) in kidney of gilthead sea bream. Further details as in legend to Table 2**

| Parameter | Density | Feeding conditions: Fed | Feeding conditions: Food deprived |
|---|---|---|---|
| GPase activity | | | |
| Total activity | Normal | 0.05 ± 0.005 | 0.05 ± 0.005 |
| ($U\,mg^{-1}$ protein) | High | 0.07 ± 0.006 | 0.06 ± 0.003 |
| % GPase *a* | Normal | 36.9 ± 2.48 | 35.9 ± 4.37 |
| | High | 28.4 ± 2.02 | 35.4 ± 2.52 |
| PK activity | | | |
| Optimal activity | Normal | 2.27 ± 0.21 | 2.26 ± 0.11 |
| ($U\,mg^{-1}$ protein) | High | 2.92 ± 0.15# | 3.45 ± 0.17# |
| Activity ratio | Normal | 0.20 ± 0.01 | 0.18 ± 0.01 |
| | High | 0.19 ± 0.01 | 0.19 ± 0.01 |
| Fructose 1,6-$P_2$ | Normal | 0.36 ± 0.02 | 0.32 ± 0.01 |
| activation ratio | High | 0.36 ± 0.03 | 0.35 ± 0.02 |
| HK activity | | | |
| Optimal activity | Normal | 0.16 ± 0.03 | 0.17 ± 0.02 |
| ($U\,mg^{-1}$ protein) | High | 0.29 ± 0.02# | 0.24 ± 0.03# |

*Further details as in legend to Table 2.

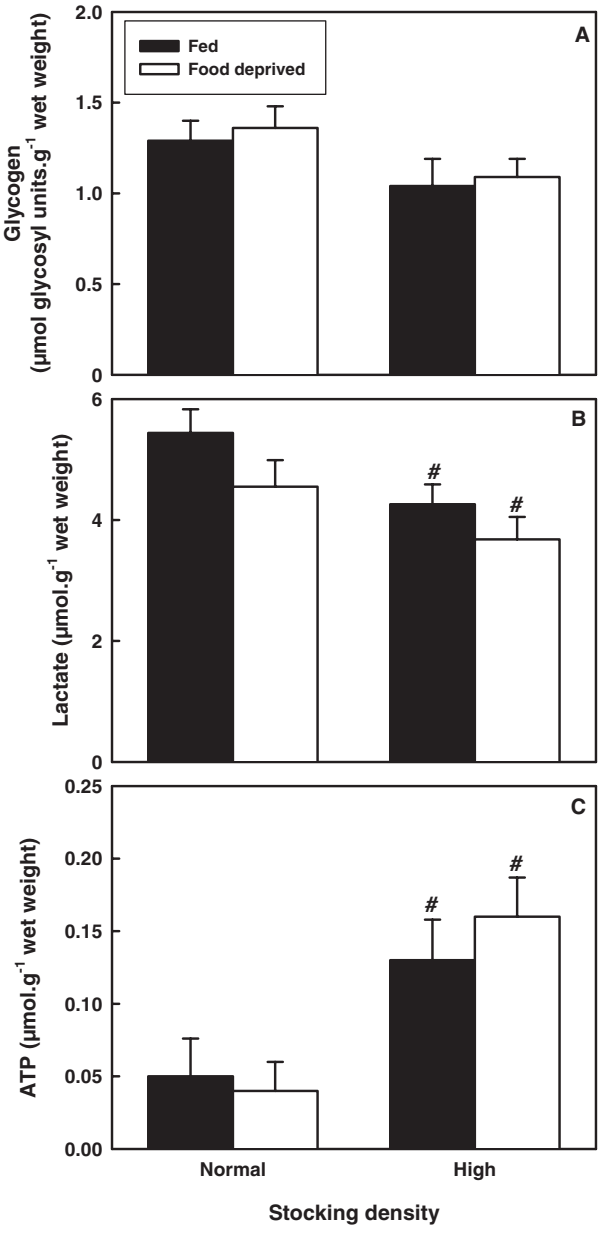


Fig. 5. Effects of high stocking density and/or food deprivation for 14 days on levels of glycogen (**A**), lactate (**B**) and ATP (**C**) in the brain of the gilthead sea bream. Further details as in legend to Fig. 1.

which can be attributed to the increase observed in the glycogenolytic potential (enhanced GPase activity) and agrees with data obtained previously in other fish species under conditions of HSD (Vijayan et al., '90; Scott-Thomas et al., '92) or confinement (Vijayan et al., '97; Trenzado et al., 2003). This enhanced mobilization of glucose from glycogen stores is also accompanied by increased glucose production through gluconeogenesis (20% increase in FBPase activity in livers of HSD fish) as well as by the 5-fold increase in plasma levels of cortisol in HSD fish. Increased gluconeogenic capacity in the liver has also been reported in other fish species under conditions of HSD (Vijayan et al., '90; Scott-Thomas et al., '92) and confinement (Vijayan et al., '97; Trenzado et al., 2003). Moreover, it is interesting to remark that a similar increase in FBPase activity is produced by cortisol treatment in the livers of the gilthead sea bream (Laiz-Carrión et al., 2002, 2003) and other species (Dziewulska-Szwajkowska et al., 2003). The enhancement of glucose production in the liver of HSD fish is not apparently directed to be used within liver since carbohydrate utilization through glycolysis and the pentose phosphate pathway are reduced based on decreased levels of metabolites (ATP, lactate) and enzyme activities (PK, G6PDH). In contrast, the capacity of liver to export glucose is increased in HSD fish as judged by the 100% increase displayed by G6Pase activity. This is the first time that glucose export capacity of the liver has been shown to be affected by HSD conditions since in the other study in which this capacity has been evaluated, no changes in this enzyme activity were noticed (Vijayan et al., '90). Cortisol could be involved in increased G6Pase activity considering the increased levels of plasma

*TABLE 5. Effects of high stocking density and/or food-deprivation for 14 days on activities of glycogen phosphorylase (GPase), 6-phosphofructo 1-kinase (PFK), hexokinase (HK), and glucose 6-phosphate dehydrogenase (G6PDH) in brain of gilthead sea bream. Further details as in legend to Table 2**

| | | Feeding conditions | |
|---|---|---|---|
| Parameter | Density | Fed | Food deprived |
| GPase activity | | | |
| Total activity | Normal | 0.33 ± 0.01 | 0.32 ± 0.02 |
| ($U\,mg^{-1}$ protein) | High | 0.36 ± 0.01$^{\sharp}$ | 0.38 ± 0.02$^{\sharp}$ |
| % GPase *a* | Normal | 43.6 ± 1.86 | 40.8 ± 2.57 |
| | High | 42.8 ± 1.08 | 39.8 ± 2.41 |
| PFK activity | | | |
| Optimal activity | Normal | 5.78 ± 0.22 | 6.20 ± 0.27* |
| ($U\,mg^{-1}$ protein) | High | 5.99 ± 0.20 | 6.70 ± 0.36* |
| Activity ratio | Normal | 0.019 ± 0.002 | 0.029 ± 0.002* |
| | High | 0.022 ± 0.002 | 0.028 ± 0.002* |
| Fructose 2,6-$P_2$ | Normal | 0.11 ± 0.01 | 0.15 ± 0.01 |
| activation ratio | High | 0.13 ± 0.02 | 0.13 ± 0.01 |
| HK activity | | | |
| Optimal activity | Normal | 1.13 ± 0.06 | 1.13 ± 0.04 |
| ($U\,mg^{-1}$ protein) | High | 1.07 ± 0.04 | 1.17 ± 0.06 |
| G6PDH activity | | | |
| Optimal activity | Normal | 0.37 ± 0.03 | 0.38 ± 0.02 |
| ($U\,mg^{-1}$ protein) | High | 0.34 ± 0.02 | 0.36 ± 0.02 |

*Further details as in legend to Table 2.

cortisol measured in HSD fish in the present study, and the known activation of G6Pase activity elicited by cortisol treatment in the carp (Dziewulska-Szwajkowska et al., 2003).

The increased capacity for exporting glucose that displayed livers of fish maintained under HSD conditions is also reflected by increased levels of glucose in plasma, in agreement with other studies under similar HSD conditions in the gilthead sea bream (Arends et al., '99; Montero et al., '99; Rotllant et al., 2000, 2001) and other fish species (Staurnes et al., '94; Ruane et al., 2002; Trenzado et al., 2003). Hyperglycemia together with decreased liver glycogen, is a common secondary response to acute stress in fishes and is attributed to the immediate effects of catecholamines on glycogenolysis and the longer-term effects of cortisol on gluconeogenesis (Arends et al., '99; Ruane et al., 2001). Considering the rise observed in plasma cortisol levels in HSD fish, the second possibility seems probable. Plasma lactate levels decreased in fish under HSD conditions in contrast with the absence of changes reported by Arends et al. ('99) in the same species during confinement, though in the later study, lactate levels were assessed only after 24 h instead of the long-term assessment in the present experiments. In other fish species, both decreases (Ruane et al., 2001) or increases (Vijayan et al., '90; Staurnes et al., '94; Reubush and Heath, '96; Ruane et al., 2002; Trenzado et al., 2003) in plasma lactate levels were noticed under conditions of HSD or confinement. The decreased levels of lactate in plasma may be related to a higher use of this metabolite for gluconeogenesis in the liver considering the 20% increase in the capacity of that pathway in livers of HSD fish. It is also possible that some of the lactate used by the liver is channelled for the replenishment of glycogen in addition to glucose production (Vijayan and Moon, '94).

In gills, HSD elicited changes that can be summarized in an apparent decrease of carbohydrate utilization as judged by decreased (30%) HK activity and lactate (50%) levels. There are no similar studies to compare with our data but considering the raised cortisol levels in plasma of HSD fish, it is interesting to remark that cortisol treatment in this species also produces a decrease in HK activity of gills (Laiz-Carrión et al., 2002, 2003). This decreased enzyme activity also coincides with elevated levels of glucose in plasma, suggesting a decrease in the capacity of gills to phosphorylate and use exogenous glucose. The absence of changes in the glycolytic potential of gills in fish under HSD conditions also agrees with the absence of changes in the same potential observed when *S. auratus* is under cortisol treatment (Laiz-Carrión et al., 2002). Altogether, gill carbohydrate metabolism appears to be redirected to an increased use of metabolic fuels other than glucose under the stress situation imposed by HSD conditions (this study) or under cortisol treatment (Laiz-Carrión et al., 2002). Confinement stress induces changes in plasma ion levels in several species (Wendelaar Bonga, '97) including the gilthead sea bream (Arends et al., '99; Rotllant et al., 2001). It has been suggested that those changes in plasma ion levels under confinement could produce an enhancement in the osmoregulatory work of gills (Wendelaar Bonga, '97; Ruane et al., '99). A hypothetical increased osmoregulatory work of gills would match with the use of alternative fuels elicited by HSD conditions.

In the kidney, the 40% increase observed in HK activity in HSD fish suggests a possible increase in the capacity for use of exogenous glucose, which has not been previously addressed in any other fish species under similar stress conditions or under cortisol treatment. The increased capacity

for use of exogenous glucose in HSD fish is apparently directed to store glucose in the form of glycogen, as demonstrated by increased glycogen levels in the kidney of fish under HSD conditions, and to be increasingly used in situ through glycolysis, as suggested by the elevation of PK activity. The significance of these changes is unclear; however, considering that kidney has an important osmoregulatory function (Beyenbach, '95), the presence of a big pool of glycogen in this tissue guarantees a fast supply of glucose to cope with the adverse conditions generated by stress in a species (like all teleost fish) without a developed homeostatic control of glycemia (Blasco et al., 2001).

In the brain, physiological conditions related to stress generally require an enhancement of the energy demand (Soengas and Aldegunde, 2002). However, HSD conditions have little impact on energy metabolism of the brain since only a slight increase in glycogenolytic potential (10%), decreased lactate (20%) and increased ATP levels (250%) were noticed in HSD fish. When comparing these results with those observed in the same species under cortisol treatment (Laiz-Carrión et al., 2002, 2003), a similar increase was observed in ATP levels, suggesting that HSD conditions are enhancing (through increased levels of cortisol in plasma) the use of those pathways involved in ATP production. Considering the absence of other changes in the metabolic potential of the brain, it seems that the stress induced by HSD conditions is not important enough to produce any other changes in brain metabolic potential of *S. auratus* when compared with cortisol treatment (Laiz-Carrión et al., 2002, 2003) or with another stress situation such as acclimation to different salinities (Sangiao-Alvarellos et al., 2003b).

Considering that HSD conditions are known to increase plasma cortisol levels in the gilthead sea bream (Arends et al., '99; Montero et al., '99; Rotllant et al., 2001; this study) and other species (Vijayan et al., '90; Rotllant and Tort, '97; Trenzado et al., 2003), the stress-related elevation of plasma cortisol observed in the present experiment could be involved as a primary response in the secondary response of mobilization of fish energy reserves (Montero et al., 2001). Moreover, several studies have shown that catecholamines rise immediately after confinement stress (Gamperl et al., '94), and this transient increase results in rapid glycogen breakdown and, consequently, elevated plasma glucose concentration (Vijayan and Moon, '94; Arends et al., '99) thus making it possible that cortisol either directly and/or indirectly with other hormones, including epinephrine, is involved in changes of carbohydrate metabolism (Reid et al., '92). In this regard, the results obtained in parameters of carbohydrate metabolism in HSD fish are similar to those reported after cortisol (Laiz-Carrión et al., 2002, 2003) or noradrenaline (Sangiao-Alvarellos et al., 2003a) treatment, suggesting the possible involvement of both endocrine systems as a primary response eliciting the secondary metabolic response observed in the tissues assessed.

### *Effects of food deprivation*

Food deprivation for 14 days of *S. auratus* under NSD conditions produced in plasma a 3-fold increase in cortisol levels similar to that addressed in comparable studies (Moon et al., '89; Vijayan et al., '96; Jorgensen et al., 2002; Pottinger et al., 2003). In the liver, food deprivation resulted in an increase in glycogenolytic potential, as judged in NSD fish by changes observed in glycogen levels and GPase activity, and a 20% increase in gluconeogenic potential. The increased potential for gluconeogenesis in the liver is in agreement with data obtained in the same species by Bonamusa et al. ('92), and in the red sea bream by Woo and Fung ('81) whereas decreased glycogen levels in the liver have been observed also in *S. auratus* during fasting (Power et al., 2000). A similar enhancement of glycogenolytic and gluconeogenic potential in the liver had been previously observed in other fish species (Foster and Moon, '91; Sheridan and Mommsen, '91; Soengas et al., '96, '98; Figueroa et al., 2000; Figueiredo-Garutti et al., 2002; Pottinger et al., 2003; Kirchner et al., 2005) and can be attributed, at least in part, to the increased cortisol levels in plasma. The increased hepatic production of glucose through gluconeogenesis and glycogenolysis in food-deprived fish was also reflected in the 45% increase of G6Pase activity in food-deprived fish, supporting an enhancement in the capacity of the liver of the gilthead sea bream for exporting glucose. These results are in agreement with the increased G6Pase activity (Caseras et al., 2002) and increased G6Pase mRNA levels (Metón et al., 2004) reported in the liver of the same species under food deprivation conditions. The important increase in this capacity in food-deprived fish may help to explain why plasma glucose levels were maintained in the present experiments in contrast with the hypoglycemia reported in others (see

Navarro and Gutiérrez, '95). Power et al. (2000) reported in the gilthead sea bream fasted for 3 weeks a hypoglycemic response, which could be attributed to a non-activation of the gluconeogenic potential compared with the present data. The fall observed in plasma lactate levels is similar to that observed during food deprivation in other species (Soengas et al., '96, '98; Vijayan et al., '96) and is probably related to the increased use of this metabolite as substrate for hepatic gluconeogenesis.

In the brain, the only important change observed after 14 days of food deprivation was the increase in glycolytic potential as judged by the 10% increase in PFK activity and the 20% decrease in lactate levels. This increased glycolytic capacity agrees with data obtained previously in the brain of food-deprived salmonids (Soengas et al., '96, '98) though not in the catfish (Tripathi and Verma, 2003). Since in the brain of salmonids, important changes were also noticed regarding glucose phosphorylating and glycogenolytic capacities (Soengas et al., '96, '98), this supports the fact that the metabolic response to food deprivation is highly species-specific (Sheridan and Mommsen, '91; Navarro and Gutiérrez, '95).

There are few studies in which the effects of food deprivation have been assessed in carbohydrate metabolism of osmoregulatory organs such as the gills and kidney (Morata et al., '82; Jürss et al., '86; Kirchner et al., 2005). Our results showed that in both tissues the more important effect of food deprivation under NSD conditions was the decrease observed in lactate levels (44% and 55% for gills and kidney, respectively). Thus, it seems that the energy demand of these important osmoregulatory organs in *S. auratus* (Sangiao-Alvarellos et al., 2003b) is maintained under food deprivation conditions and the major pathways involved in energy production in both tissues (Mommsen, '84; Mommsen et al., '85) are not affected to preserve their functionality. The decreased lactate levels could be related in gills to an increased use as an oxidative fuel (Mommsen, '84), whereas in the kidney, it could be related to an increased capacity of the gluconeogenic pathway (Mommsen et al., '85). The increase in gluconeogenic potential in the kidney would be in agreement with the increase in plasma cortisol levels in the present study and with the reported increased activities of PEPCK (Morata et al., '82) and glutamate dehydrogenase (Sánchez-Muros et al., '98) in the kidney of food-deprived rainbow trout, though not with the absence of changes in FBPase activity and expression observed in the kidney of food-deprived rainbow trout (Kirchner et al., 2005).

### *Interaction between HSD and feeding conditions*

The combined effect of HSD and food deprivation on energy metabolism has not been assessed in any previous study in teleost fish. In the present study, the increased levels of cortisol and glucose in plasma elicited by HSD conditions are increased even when HSD fish are under food deprivation. Thus, an additional 50% increase in cortisol levels and an additional 14% increase in plasma glucose were noticed when comparing with fed fish under HSD conditions. Similar increases of plasma glucose have been observed under the combined action of food deprivation and handling (Vijayan and Moon, '92; Reubush and Heath, '96) as well as food deprivation and PCB exposure (Jorgensen et al., 2002), whereas a similar effect has been previously observed for plasma cortisol levels under food deprivation and handling (Vijayan and Moon, '92), and food deprivation and PCB exposure (Jorgensen et al., 2002). The source of the increased glucose levels appears to be of hepatic nature since the interaction between HSD conditions and food deprivation produces a further increase in the glycogenolytic potential in the liver, i.e., HSD stress mobilizes 250% more hepatic glycogen stores in food-deprived than in fed fish in a way comparable with studies using handling stress (Vijayan and Moon, '92; Reubush and Heath, '96). Considering that (1) hepatic glycogen stores are utilized for immediate energy requirements by fish in stressful situations and (2) the potential for glycogen breakdown by hepatocytes is lower in food-deprived fish (Vijayan et al., '93), it seems that the interaction of HSD conditions with food deprivation induce a change in the capacity of the liver in food-deprived fish to mobilize glycogen. The other source of glucose production in the liver, i.e., through gluconeogenesis, does not appear to be further activated since FBPase activity in food-deprived fish under HSD conditions was similar to that of fed fish. Another significant change was that ATP levels under HSD conditions also decreased to a greater extent (3-fold) in food-deprived than in fed fish, reinforcing the model of enhanced glucose production from glycogen stores to be exported to other tissues rather than being used in situ to raise ATP levels. This increased exporting capacity in

food-deprived fish under HSD conditions agrees well with the increase also displayed by G6Pase activity in food-deprived vs. fed fish. As a whole, the interaction between food deprivation and HSD conditions produces an enhancement in glycogen mobilization of liver stores to produce readily available glucose in plasma. On the other hand, feeding state did not affect the response of plasma lactate levels to HSD stress, in agreement with data obtained by Vijayan and Moon ('92) and Reubush and Heath ('96) comparing fed and food-deprived fish after handling stress.

The interaction between both factors elicited interesting changes in gills since the glycogenolytic potential, which was not altered by food deprivation or HSD conditions separately, produced a clear decrease of 40% vs. fed fish in glycogen levels only when both parameters act simultaneously. It seems that the cumulative effects of both stressors produce an enhancement in the energy demand of the gills that would be related to correct the accompanying hydromineral balance (Barton and Iwama, '91) due to the additional metabolic load imposed by food deprivation to HSD stress. In contrast, the interaction of HSD conditions with food deprivation did not alter significantly any metabolic parameter assessed in the kidney and brain, suggesting that these tissues are less responsive to the cumulative effects of stressors on energy metabolism.

The results of the present study demonstrate that HSD conditions elicit important primary (increased cortisol) and secondary (metabolic changes) stress responses in the gilthead sea bream. Some of the changes in energy metabolism were confirmatory of previous studies (several hepatic parameters), whereas in others they provide new information regarding metabolic adjustments to cope with HSD conditions in the liver, gills, kidney and brain. On the other hand, food deprivation also resulted in primary and secondary stress responses. Some of the changes addressed in the liver and brain were confirmatory of previous studies whereas new information was obtained for the liver, gills and kidney. Furthermore, the results obtained provide, for the first time, information indicating that food deprivation in fish increased the sensitivity to the stress induced by HSD conditions compared with the fed controls. This is reflected as a primary response by the further increase noticed in plasma cortisol levels of food-deprived fish under HSD conditions. Moreover, several interactive effects were noticed in the secondary responses observed in the liver, in agreement with similar models raised using handling stress (Vijayan and Moon, '92; Reubush and Heath, '96) or PCB exposure (Jorgensen et al., 2002). In this way, it is interesting to note that several authors have shown that the response of fish to several stressors, in terms of cortisol and metabolism was cumulative (Barton et al., '86; Davis and Schreck, '97; Rotllant and Tort, '97), lending support for a cumulative effect of both stressors on parameters assessed in the present experiments on carbohydrate metabolism in the liver, with no major cumulative effects being noticed in the other tissues, thus providing information regarding reallocation of metabolic energy to cope with simultaneous stressors.

## ACKNOWLEDGMENT

This work was supported by Grants BOS2001-4031-C02-01 (Ministerio de Ciencia y Tecnología, Spain and FEDER) to J.M.M., and Grants BOS2001-4031-C02-02 and VEM2003-20062 (Ministerio de Ciencia y Tecnología, Spain and FEDER), PGIDT01PXI30113PR and PGIDT04P XIC31208PN (Xunta de Galicia, Spain) and c46164102 (Universidade de Vigo) to J.L.S. S.S-A. was recipient of a predoctoral fellowship from the Xunta de Galicia. R.L-C. was recipient of a MIT-2 predoctoral fellowship from the Ministerio de Ciencia y Tecnología. The authors wish to thank Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish.

## LITERATURE CITED

Arends RJ, Mancera JM, Muñoz JL, Wendelaar Bonga SE, Flik G. 1999. The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. J Endocrinol 163:149–157.

Arends RJ, Rotllant J, Metz J, Mancera JM, Wendelaar Bonga SE, Flik G. 2000. α-Melanocyte stimulating hormone acetylation in the pituitary gland of the sea bream (*Sparus aurata* L.) in response to different backgrounds, confinement and air exposure. J Endocrinol 166:427–435.

Barton BA, Iwama GK. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu Rev Fish Dis 129:3–26.

Barton BA, Schreck CB, Sigismondi LA. 1986. Multiple acute disturbances evoke cumulative physiological stress responses in juvenile chinook salmon. Trans Am Fish Soc 115:245–251.

Beyenbach KW. 1995. Secretory electrolyte transport in renal proximal tubules of fish. In: Wood CM, Shuttleworth TJ, editors. Fish physiology, Vol XIV, Ionorregulation: cellular and molecular approaches. New York: Academic Press. p 85–106.

Blasco J, Marimon I, Viaplana I, Fernández-Borrás J. 2001. Fate of plasma glucose in tissues of brown trout *in vivo*: effects of fasting and glucose loading. Fish Physiol Biochem 24:247–258.

Bonamusa L, García de Frutos P, Fernández F, Baanante IV. 1992. Nutritional effects on key glycolytic-gluconeogenic enzyme activities and metabolite levels in the liver of the teleost fish *Sparus aurata*. Mol Mar Biol Biotechnol 1:113–125.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Caseras A, Metón I, Vives C, Egea M, Fernández F, Baanante IV. 2002. Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (*Sparus aurata*). Br J Nutr 88:607–614.

Collins AL, Anderson TA. 1997. The influence of changes in food availability on the activation of key degradative and metabolic enzymes in the liver and epaxial muscle of the golden Perch. J Fish Biol 50:1158–1165.

Davis LE, Schreck CB. 1997. The energetic response to handling stress in juvenile coho salmon. Trans Am Fish Soc Symp 126:248–258.

Dziewulska-Szwajkowska D, Lozinska-Gabska M, Adamowicz A, Wojtaszek J, Dzugaj A. 2003. The effect of high dose of cortisol on glucose-6-phosphatase and fructose-1, 6-bisphosphatase activity, and glucose and fructose 2-6-bisphosphate concentration in carp tissues (*Cyprinus carpio* L.). Comp Biochem Physiol 135B:485–491.

Figueiredo-Garutti ML, Navarro I, Capilla E, Souza RHS, Moraes G, Gutiérrez J, Vicentini-Paulino MLM. 2002. Metabolic changes in *Brycon cephalus* (Teleostei, Characidae) during post-feeding and fasting. Comp Biochem Physiol 132A:467–476.

Figueroa RI, Rodríguez-Sabarís R, Aldegunde M, Soengas JL. 2000. Effects of food deprivation on 24h-changes in brain and liver carbohydrate and ketone body metabolism of rainbow trout. J Fish Biol 57:631–646.

Foster GD, Moon TW. 1991. Hypometabolism with fasting in the yellow perch: a study of enzymes, hepatocyte metabolism, and tissue size. Physiol Zool 64:259–275.

Gamperl AK, Vijayan MM, Boutilier RG. 1994. Epinephrine, norepinephrine, and cortisol concentrations in cannulated seawater-acclimated rainbow trout (*Oncorhynchus mykiss*) following black box confinement and epinephrine injection. J Fish Biol 45:313–324.

Jorgensen EH, Vijayan MM, Aluru N, Maule AG. 2002. Fasting modifies Aroclor 1254 impact on plasma cortisol, glucose and lactate responses to handling disturbance in Arctic charr. Comp Biochem Physiol 132C:235–245.

Jürss K, Bittorf T, Vökler T. 1986. Influence of salinity and food deprivation on growth, RNA/DNA ratio and certain enzyme activities in rainbow trout (*Salmo gairdneri* Richardson). Comp Biochem Physiol 83B:425–433.

Keppler D, Decker K. 1974. Glycogen. Determination with amyloglucosidase. In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York: Academic Press. p 1127–1131.

Kirchner S, Seixas P, Kaushik S, Panserat S. 2005. Effects of low protein intake on extra-hepatic gluconeogenic enzyme expression and peripheral glucose phosphorylation in rainbow trout (*Oncorhynchus mykiss)*. Comp Biochem Physiol 140B:333–340.

Laiz-Carrión R, Sangiao-Alvarellos S, Guzmán JM, Martín del Rio MP, Soengas JL, Mancera JM. 2002. Energy metabolism in fish tissues related to osmoregulation and cortisol action. Fish Physiol Biochem 27:179–188.

Laiz-Carrión R, Martín del Río MP, Míguez JM, Mancera JM, Soengas JL. 2003. Influence of cortisol on osmoregulation and energy metabolism in gilthead sea bream *Sparus aurata*. J Exp Zool A 298:105–118.

Lefrançois C, Claireaux G, Mercier C, Aubin J. 2001. Effect of density on the routine metabolic expenditure of farmer rainbow trout (*Oncorhynchus mykiss*). Aquaculture 195: 269–277.

Metón I, Caseras A, Fernández F, Baanante IV. 2004. Molecular cloning of hepatic glucose-6-phosphatase catalytic subunit from gilthead sea bream (*Sparus aurata*): response of its mRNA levels and glucokinase expression to refeeding and diet composition. Comp Biochem Physiol 138B:145–153.

Mommsen TP. 1984. Metabolism of the fish gill. In: Hoar WS, Randall DJ, editors. Fish physiology, Vol. XB. New York: Academic Press. p 203–238.

Mommsen TP, Walsh PJ, Moon TW. 1985. Gluconeogenesis in hepatocytes and kidney of Atlantic salmon. Mol Physiol 8:89–100.

Montero D, Izquierdo MS, Tort L, Robaina L, Vergara JM. 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol Biochem 20:53–60.

Montero D, Robaina LE, Socorro J, Vergara JM, Tort L, Izquierdo MS. 2001. Alteration of liver and muscle fatty acid composition in gilthead seabream (*Sparus aurata*) juveniles held at high stocking density and fed an essential fatty acid defficient diet. Fish Physiol Biochem 24:63–72.

Moon TW, Foster GD, Plisetskaya EM. 1989. Changes in peptide hormones and liver enzymes in the rainbow trout deprived of food for 6 weeks. Can J Zool 67:2189–2193.

Morata P, Vargas AM, Sánchez-Medina F, García F, Cardenete G, Zamora S. 1982. Evolution of gluconeogenic enzyme activities during starvation in liver and kidney of the rainbow trout (*Salmo gairdneri*). Comp Biochem Physiol 71B:65–70.

Navarro I, Gutiérrez J. 1995. Fasting and starvation. In: Hochachka PW, Mommsen TP, editors. Metabolic biochemistry, biochemistry and molecular biology of fishes, Vol. 4. Amsterdam: Elsevier. p 393–434.

Pottinger TG, Rand-Weaver M, Sumpter JP. 2003. Overwintering fasting and re-feeding in rainbow trout: plasma growth hormone and cortisol levels in relation to energy mobilisation. Comp Biochem Physiol 136B:403–417.

Power DM, Melo J, Santos CRA. 2000. The effect of food deprivation and refeeding on the liver, thyroid hormones and transthyretin in sea bream. J Fish Biol 56:374–387.

Reid SD, Moon TW, Perry SF. 1992. Rainbow trout hepatocyte beta-adrenoceptors, catecholamine responsiveness, and effects of cortisol. Am J Physiol 262:R794–R799.

Reubush KJ, Heath AG. 1996. Metabolic responses to acute handling by fingerling inland and anadromous striped bass. J Fish Biol 49:830–841.

Rotllant J, Tort L. 1997. Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. J Fish Biol 51: 21–28.

Rotllant J, Arends RJ, Mancera JM, Flik G, Wendelaar Bonga SE, Tort L. 2000. Inhibition of HPI axis response to stress in gilthead sea bream (*Sparus aurata*) with physiological plasma levels of cortisol. Fish Physiol Biochem 23:13–22.

Rotllant J, Balm PHM, Pérez-Sánchez J, Wendelaar Bonga SE, Tort L. 2001. Pituitary and interrenal function in gilthead sea bream (*Sparus aurata* L., Teleostei) after handling and confinement stress. Gen Comp Endocrinol 121:333–342.

Ruane NM, Wendelaar Bonga SE, Balm P. 1999. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. Gen Comp Endocrinol 115:210–219.

Ruane NM, Huisman EA, Komen J. 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. J Fish Biol 59:1–12.

Ruane NM, Carballo EC, Komen J. 2002. Increased stocking density influences the acute physiological stress response of common carp *Cyprinus carpio* (L.). Aquacult Res 33:777–784.

Sánchez-Muros MJ, García-Rejón L, García-Salguero L, de la Higuera M, Lupiáñez JA. 1998. Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptative response to starvation and high-protein, carbohydrate-free diet to glutamate dehydrogenase and alanine aminotransferase kinetics. Int J Biochem 30: 55–63.

Sangiao-Alvarellos S, Bouça P, Míguez JM, Soengas JL. 2003a. Intracerebroventricular injections of noradrenaline affect brain energy metabolism of rainbow trout. Physiol Biochem Zool 76:663–671.

Sangiao-Alvarellos S, Láiz-Carrión R, Guzmán JM, Martín del Rio MP, Míguez JM, Mancera JM, Soengas JL. 2003b. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. Am J Physiol 285:R897–R907.

Sangiao-Alvarellos S, Lapido M, Míguez JM, Soengas JL. 2004. Effects of central administration of arginine vasotocin on monoaminergic neurotransmitters and energy metabolism of rainbow trout brain. J Fish Biol 64:1313–1329.

Sangiao-Alvarellos S, Guzmán JM, Laiz-Carrión R, Martín del Río MP, Míguez JM, Mancera JM, Soengas JL. 2005. Actions of 17ß-estradiol on carbohydrate metabolism in liver, gills and brain of gilthead sea bream *Sparus auratus* during acclimation to different salinities. Mar Biol 146:607–617.

Scott-Thomas DAF, Ballantyne JS, Leatherland JF. 1992. Interactive effects of high stocking density and triiodothyronine-administration on aspects of the *in vivo* intermediary metabolism and in vitro hepatic response to catecholamine and pancreatic hormone stimulation in brook charr, *Salvelinus fontinalis*. J Exp Zool 263:68–82.

Sheridan MA, Mommsen TP. 1991. Effects of nutritional state on in vivo lipid and carbohydrate metabolism of Coho Salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 81: 473–483.

Soengas JL, Aldegunde M. 2002. Energy metabolism of fish brain. Comp Biochem Physiol 131B:271–296.

Soengas JL, Strong EF, Fuentes J, Veira JAR, Andrés MD. 1996. Food deprivation and refeeding in Atlantic salmon, *Salmo salar*: effects on brain and liver carbohydrate and ketone bodies metabolism. Fish Physiol Biochem 15: 491–511.

Soengas JL, Strong EF, Andrés MD. 1998. Glucose, lactate, and ß-hydroxybutyrate utilization by rainbow trout brain: changes during food deprivation. Physiol Zool 71:285–293.

Staurnes M, Sigholt T, Pedersen HP, Rustad T. 1994. Physiological effects of stimulated high-density transport of Atlantic cod (*Gadus morhua*). Aquaculture 119:381–391.

Tintos A, Miguez JM, Mancera JM, Soengas JL. 2005. Development of a microtitre palte indirect ELISA for measuring cortisol in teleost fish, and evaluation of stress responses in rainbow trout and gilthead sea bream. J Fish Biol In press.

Trenzado CE, Carrick TR, Pottinger TG. 2003. Divergence of endocrine and metabolic responses to stress in two rainbow trout lines selected for differing cortisol responsiveness to stress. Gen Comp Endocrinol 133:332–340.

Tripathi G, Verma P. 2003. Starvation-reduced impairment of metabolism in a freshwater catfish. Z Naturforsch C 58: 446–451.

Vijayan MM, Moon TW. 1992. Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (*Oncorhynchus mykiss*). Can J Fish Aquat Sci 49:2260–2266.

Vijayan MM, Moon TW. 1994. The stress response and the plasma disappearance of corticosteroid and glucose in a marine teleost, the sea raven. Can J Zool 72:379–386.

Vijayan MM, Ballantyne JS, Leatherland JF. 1990. High stocking density alters the energy metabolism of brook charr, *Salvelinus fontinalis*. Aquaculture 88:371–381.

Vijayan MM, Maule AG, Schreck CB, Moon TW. 1993. Hormonal control of hepatic glycogen metabolism in food deprived, continuously swimming coho salmon (*Oncorhynchus kisutch*). Can J Fish Aquat Sci 50:1676–1682.

Vijayan MM, Morgan JD, Sakamoto T, Grau EG, Iwama GK. 1996. Food-deprivation affects seawater acclimation in tilapia: hormonal and metabolic changes. J Exp Biol 199: 2467–2475.

Vijayan MM, Pereira C, Grau EG, Iwama GK. 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. Comp Biochem Physiol 116C:89–95.

Wagner EJ, Bosakowski T, Intelmann S. 1997. Combined effects of temperature and high pH on mortality and the stress response of rainbow trout after stocking. Trans Am Fish Soc Symp 126:985–998.

Wendelaar Bonga SE. 1997. The stress response in fish. Physiol Rev 77:591–625.

Woo NYS, Fung ACY. 1981. Biology of red sea bream *Chrysophrys major*. 4. Metabolic effects of starvation at low temperature. Comp Biochem Physiol A 69:461–466.