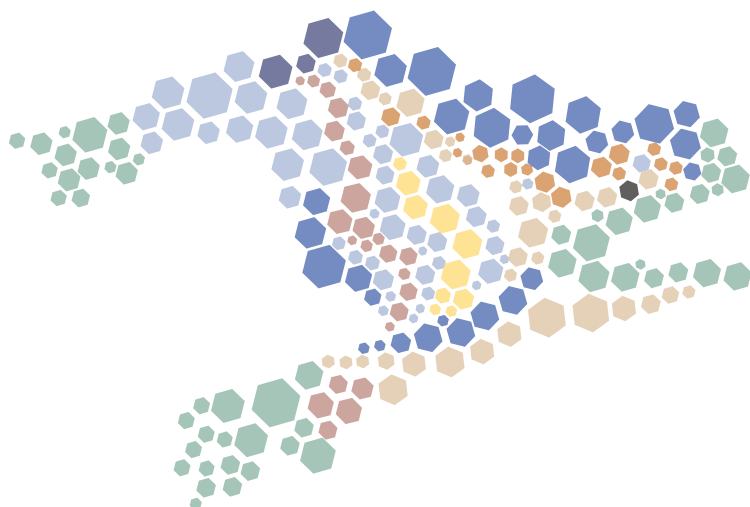


# Advances in Comparative Endocrinology

Vol. VII



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## EFFECTS OF NUTRITIONAL STATUS ON PLASMA LEPTIN LEVELS AND IN VITRO REGULATION OF ADIPOCYTE LEPTIN EXPRESSION AND SECRETION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The aim of the study was to explore the modulatory effect of adiposity on plasma leptin, as well as the regulatory role of hormones and nutrients on the expression and secretion of leptin from adipocytes in rainbow trout. Fish were fed a high-energy (HE) diet at two different ration levels, *ad libitum* (AL group) or 25% of satiation (RE group) for eight weeks. RE fish had significantly reduced growth and adipose tissue weight in comparison to AL fish, but increased plasma leptin levels ( $p=0.022$ ). Interestingly, and contrary to mammals, plasma leptin was negatively correlated with adipose tissue mass and mesenteric fat index, suggesting that plasma leptin in fish can still convey information on the energy status of the body. Next, isolated adipocytes from fish on both ration levels and fish fed a regular diet were used to study *in vitro* the factors that may regulate leptin expression and secretion. Adipocytes were treated with insulin, ghrelin, leucine or eicosapentaenoic acid (EPA) or left untreated (control). In regular-diet adipocytes, both insulin and ghrelin increased leptin secretion dose-dependently ( $p=0.002$  and  $p=0.033$ , respectively), and insulin showed a tendency to reduce leptin expression. Results from the HE diet fish supported the findings on plasma leptin levels, with significantly higher leptin secretion in control adipocytes from RE compared to AL fish ( $p=0.022$ ). Furthermore, no significant effects on treatment were observed, neither in leptin expression nor secretion, for the AL group. In RE fish, leptin expression remained unchanged, whereas leptin secretion was significantly reduced by leucine ( $p=0.025$ ), and had a tendency to increase after insulin treatment and to decrease with EPA. These data indicate that regulation of leptin secretion from adipocytes occurs mainly at a post-transcriptional level in rainbow trout and is modulated by the nutritional history of the fish.

### Introduction

In mammals, leptin is primarily secreted by adipose tissue, the plasma leptin levels are known to correlate with adipose tissue mass, and it is considered that leptin works as an adiposity signal (1). Leptin secretion from adipocytes is stimulated by anabolic signals (i.e. food consumption, insulin, ghrelin, leucine and EPA), whereas it is inhibited by catabolic signals (i.e. fasting or growth hormone) (2). In the present study, we have explored in rainbow trout a) if plasma leptin acts also as an adiposity signal and b) the possible nutritional and/or hormonal regulators of leptin expression and secretion in isolated adipocytes.

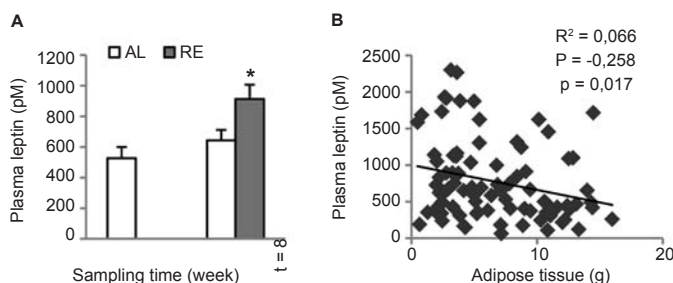
## Materials and Methods

Adult rainbow trout were fed *ad libitum* (AL) (n=44) or with a 25% of that ration (Restricted; RE) (n=42) with a commercial high-energy (HE) diet for eight weeks to obtain fish with two different levels of adiposity. Biometrics and plasma samples were obtained at time 0 (t=0) and at the end of the trial (t=8) to analyze plasma leptin by radioimmunoassay (RIA) and metabolites (glucose, triglycerides; TG and free fatty acids; FFA) using commercial kits. Visceral adipose tissue from fish at both ration levels, as well from regular diet fish, was excised and adipocytes were isolated as described by Albalat et al. (3). Around 2 million cells were incubated for 3 h with; a) different doses of insulin (I) (10 and 100 nM), ghrelin (G) (0.1, 1 and 10 nM) or left untreated (control; C) for the regular diet adipocytes (n=5-6) and, b) Insulin (Ins) 100 nM, ghrelin (Ghrel) 10 nM, leucine (Leu) 5mM or eicosapentaenoic acid (EPA) 100  $\mu$ M or left untreated (control; Ctrl) (n=6-7 with 2-4 replicates). After incubation, the medium was used to analyze leptin secretion by RIA, and the cells to analyze by real time quantitative PCR leptin A1 (LepA1) mRNA expression.

## Results and Discussion

**1. Biometrics.** AL fish had significantly increased growth and adipose tissue weight in comparison to RE fish after eight weeks of feeding on the HE diet (-47% and -52%, respectively;  $p < 0.01$ ); an also in comparison with t=0, but adipose tissue weight of RE fish did not change with time. These results corroborate the fact that the experimental design used was effective in generating two groups of fish with clearly differentiated nutritional status and fat reserves.

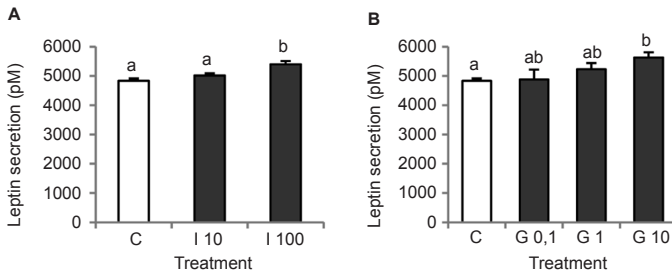
**2. Plasma parameters.** At t=8, plasma glucose remained unchanged, TG increased in both groups, but FFA increased in AL fish only (data not shown). Plasma leptin levels were higher in RE fish in comparison with AL fish ( $p = 0.022$ ) (Fig. 1A). This supports the hypothesis by Fuentes et al. (4), who suggested that increased leptin levels may be linked to a survival behavior in fish, which experience naturally prolonged periods of food shortage, lowering appetite and limiting energy-wasting foraging activity.



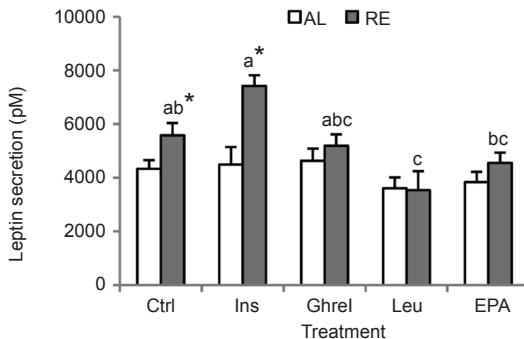
**Figure 1.** (A) Plasma leptin levels in *ad libitum* (AL) and feed restricted (RE) rainbow trout. Data at different sampling times (week t=0 and t=8) are shown as mean  $\pm$  SEM. (n=8 for t=0 and n=42-43 for t=8). No differences were observed between sampling times within the AL or RE group. Asterisk indicates significant differences between groups at t=8 ( $p < 0.05$ ). (B) Correlation between plasma leptin and adipose tissue weight of AL and RE rainbow trout at week t=8.  $R^2$  of the linear regression and correlation coefficient (P) and p value (p) from the Pearson correlation are indicated.

Moreover, contrary to the case of mammals, plasma leptin in rainbow trout was significantly negatively correlated with adipose tissue mass (Fig. 1B), mesenteric fat index and liver weight (data not shown), indicating that plasma leptin might act as an endocrine signal of adiposity in rainbow trout.

**3. Leptin expression and secretion in isolated adipocytes.** Leptin secretion from isolated adipocytes of fish fed a regular diet increased dose-dependently after incubation with insulin or ghrelin ( $p=0.002$  and  $p=0.033$ , respectively) (Fig. 2A and 2B). However, LepA1 expression did not change upon treatment (data not shown), thus suggesting that regulation of leptin secretion from adipocytes in rainbow trout occurs mainly at a post-transcriptional level.



**Figure 2.** Leptin secretion into the medium in isolated adipocytes from rainbow trout fed a regular diet after (A) insulin or (B) ghrelin treatment. Adipocytes were left untreated (C) or treated either with insulin at 10 or 100 nM (I10 or I100) or ghrelin at 0.1, 1 or 10 nM (G0.1, G1 or G10) for 3 h. Data are shown as mean  $\pm$  SEM ( $n=5-6$ ). Different letters indicate significant differences between treatments ( $p<0.05$ ).



**Figure 3.** Leptin secretion in *ad libitum* (AL) and feed restricted (RE) rainbow trout isolated adipocytes. Adipocytes were left untreated (Ctrl) or treated either with insulin 100 nM (Ins), ghrelin 10 nM (Ghrel), leucine 5 mM (Leu) or EPA 100  $\mu$ M (EPA) for 3 h. Data are shown as mean  $\pm$  SEM ( $n=6-7$  with 2-4 treatment replicates per group). Different capital or lower case letters indicate significant differences between treatments within the AL or RE group, respectively, and asterisks indicate significant differences between groups within treatments ( $p<0.05$ ).

Leptin secretion from the adipocytes of the HE diet fish supported the findings on circulating plasma leptin, with significantly higher leptin levels in the media of



control adipocytes from RE than AL fish ( $p=0.022$ ), as well as in the media of insulin-treated adipocytes from RE fish ( $p=0.005$ ) (Fig. 3). Thus, indicating that RE adipocytes retained some kind of metabolic memory. In AL fish, leptin secretion was not modified after any treatment, suggesting they might have become unresponsive. In RE fish, adipocytes incubated with anabolic hormones such as insulin, showed an increase in leptin secretion, in line with the results observed in regular diet adipocytes. Furthermore, leptin secretion in RE adipocytes was significantly reduced by leucine in comparison with the control treatment ( $p=0.025$ ) (Fig. 3) and a similar trend was observed with EPA. We might speculate that these nutrient increments could be signaled by the fish as food availability, causing a change in the metabolism by decreasing leptin secretion, which in turn induces the animal towards a feeding behavior. On the other hand, LepA1 expression did not change in response to any treatment, neither in AL nor RE isolated adipocytes (data not shown); thus supporting a post-transcriptional regulation.

Altogether, our findings suggest that the physiological role of leptin is linked to the degree of adiposity in rainbow trout and support the previous hypothesis about leptin regulating food intake and energy expenditure. Furthermore, leptin seems to be a player under the influence of other important metabolic/appetite regulating hormones such as insulin and ghrelin, which modulate leptin secretion; overall suggesting that interactions between these three hormones are crucial for the regulation of metabolism and energy balance. Also, our data reveal several aspects of the underlying mechanisms regulating leptin secretion from adipocytes in this species, which can be related to the nutritional status of the fish. Finally, the present work shows for the first time a negative correlation between plasma leptin and adipose tissue mass in rainbow trout and provides further evidence that trout fed *ad libitum* with HE diets produce excessive fat accumulation that may eventually affect the animals' health and the quality of the aquaculture product.

## Acknowledgements

Supported by funds from the MICINN (AGL2010-17324 and AGL2011-24961), the Catalanian Government (2009SGR-00402), the XRAq and by funds from the 7th Framework Program of the European Union (project LIFECYCLE FP7-222719).

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## PROPYLTHIOURACIL TREATMENT AND SALINITY TRANSFER ALTERED 5'-DEIODINATION ACTIVITY IN GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Outer ring deiodination (ORD) activity is tightly regulated by iodothyronines in mammals. In fish, discrete features of deiodinases make the whole picture much more complicated and, as a result, the regulation of ORD activity after alterations in the thyroidal status of the animal seems to be distinctive when comparing different species. In this study, we altered the thyroidal status of seawater-acclimated gilthead sea bream specimens inhibiting thyroid hormone secretion by PTU treatment for 36 days, and after that we abruptly submitted specimens for 8 days to different environmental salinities (5, 40 and 55 ppt), an environmental factor known to alter the thyroidal status in this species. Specimens treated with PTU showed a better growth; however plasma metabolite concentrations did not significantly differ between groups. This indicates that both groups, which represent two different thyroidal conditions, can reorganize their energy metabolism. Gill Na/K-ATPase (NKA) activity seems to be higher in the PTU group when submitted to hyperosmotic environments. Gill and kidney ORD activity, when measured by incubation of those tissues with T<sub>4</sub>, increased its values after 8 days of acclimation to different environmental salinities in PTU-groups. Our results show that ORD in osmoregulatory organs is sensitive to the hypo-thyroidal status induced by PTU. However, no apparent relation between ORD activities and enzyme NKA was found. This suggests that the regulation of NKA activity in gilthead sea bream does not follow the same pattern as in mammals, where changes in the thyroidal status affect this enzyme.

### Introduction

The thyroid system is constituted by many elements such as thyroid hormones (THs), deiodination enzymes, carrier proteins and membrane transporters among others (1). Triiodothyronine (T<sub>3</sub>) is the biologically active hormone in fish, but the thyroid follicles secrete mainly thyroxine (T<sub>4</sub>) which is converted to T<sub>3</sub> by two enzymes, D1 and D2, with outer-ring deiodination (ORD) activity (2). THs play many roles in animal metabolism, reproduction, metamorphosis or even salinity acclimation (3, 4). At different salinity environments, gilthead sea bream (*Sparus aurata*) modifies its thyroid system presenting a hyperthyroid state in hyposmotic environments while hypothyroidism is shown at high salinity conditions (5). It is known that hyperthyroidism suppresses D1 and D2 activities and mRNA expressions, while hypothyroidism increases them in fish (2). These changes in the thyroid system are thought to modify the metabolic pathways and thus, many *in vivo* studies have been focused on the actions exerted by anti-thyroid drugs such as

6-N-propyl-2-thiouracil (PTU), with different results depending on the species or ways of administering the drug (6, 7). For this reason, we studied the effects of PTU on the thyroid system of *S. aurata* when submitted to different salinity conditions.

## Material and Methods

Juveniles of gilthead sea bream (*S. aurata*) ( $n=160$ ,  $13.6 \pm 0.1$  cm length and  $43.3 \pm 0.6$  g weight) were maintained in four truncated-cone 1000 L tanks at 40 ppt, 19 °C and natural photoperiod (spring in the south of Spain). Two of them were fed with a control diet while the others were fed with the same diet supplemented with 2-propylthiouracil (Sigma, P-3755) in a dose of 5 mg PTU ( $\text{Kg fish}^{-1} \text{ day}^{-1}$ ) as described elsewhere (7). After 36 days, 8 animals per group were sampled and the others were submitted to different environmental salinities (5, 40 and 55 ppt). After 8 days, plasma, gills and kidney samples were collected. Fork length, wet weight, plasma metabolites, and gill and kidney Na/K-ATPase as well as T4-ORD activities were measured (4).

## Results and Discussion

Gilthead sea bream juveniles fed with a PTU complement exhibit higher growth than the control group, although both groups do not show significant differences in plasma metabolites, indicating an energy reallocation (Table 1).

**Table 1.** Effects of PTU after 36 days of treatment in *S. aurata*. (Mean $\pm$ SEM,  $n=8$ ). \* indicate differences between groups (Student-T test,  $p<0.05$ ).

| Group   | Length (cm)     | Weight (g)      | Gill Na/K-ATPase act. | Plasma lactate (mM) | Plasma free amino acids (mM) |
|---------|-----------------|-----------------|-----------------------|---------------------|------------------------------|
| Control | 16.0 $\pm$ 0.1  | 77.0 $\pm$ 1.7  | 11.5 $\pm$ 0.9        | 0.9 $\pm$ 0.1       | 19.3 $\pm$ 1.2               |
| PTU     | 16.6 $\pm$ 0.2* | 88.2 $\pm$ 3.6* | 15.0 $\pm$ 0.9*       | 0.7 $\pm$ 0*        | 13.7 $\pm$ 1.6*              |

Gills and kidney are considered the most important osmoregulatory tissues in fish and NKA activity is usually studied as a key role enzyme in this process (4). After 36 days of treatment, there were no differences between the kidney NKA activities, or in the gills and kidney T4-ORD activities of both groups (data not shown). This could indicate that gilthead sea bream fed with PTU have an increased metabolism which makes the animals grow more with few changes in their plasma metabolites. This could increase the aerobic respiration and therefore, gill NKA activity.

As PTU affects the metamorphic pathways and presents inhibitory effects on larval development (6) and decreased THs levels (8), its metabolic effects will depend on the developmental stage of the fish. PTU-induced hypothyroidism does not affect NKA activity in freshwater tilapia (8), while the present study demonstrates that PTU increases not only this enzymatic activity in gills or kidney, but also T4-ORD activity in both tissues in gilthead sea bream (Table 2).

Nevertheless, the relationships between those two enzymatic activities are not clear within the present study, suggesting different pathways of regulation in fish

and mammals. In the latter, changes in the thyroidal status affect this enzyme. Further studies will be necessary to fully understand this process, such as mRNA expression of the different counterparts or plasmatic levels of THs or cortisol, another osmoregulatory key hormone.

**Table 2.** Effects of PTU and environmental salinity on Na/K-ATPase ( $\mu\text{mol ADP mg prot}^{-1}\text{h}^{-1}$ ) and T4-ORD ( $\text{fmol } \mu\text{g}^{-1} \text{ min}^{-1}$ ) activities in *S. aurata*. (Mean $\pm$ SEM, n=8). Different letters indicate statistical differences among groups (Two way ANOVA followed by Tukey's posthoc test,  $p<0.05$ ).

| Group          | Control          |                   |                   | PTU               |                   |                  |
|----------------|------------------|-------------------|-------------------|-------------------|-------------------|------------------|
| Salinity (ppt) | 5                | 40                | 55                | 5                 | 40                | 55               |
| Gill NKA       | 8.1 $\pm$ 0.9 E  | 13.3 $\pm$ 1.4 CD | 19.7 $\pm$ 1.4 BC | 9.7 $\pm$ 1.1 DE  | 23.9 $\pm$ 1.2 AB | 33.1 $\pm$ 4.3 A |
| Kidney NKA     | 16.6 $\pm$ 1.5 A | 9.3 $\pm$ 0.5 B   | 14.5 $\pm$ 1.1 AB | 13.2 $\pm$ 1.5 AB | 15.2 $\pm$ 1.2 A  | 15.1 $\pm$ 1.3 A |
| Gill T4-ORD    | 212 $\pm$ 42 BC  | 100 $\pm$ 20 C    | 243 $\pm$ 35 ABC  | 364 $\pm$ 35 A    | 293 $\pm$ 30 AB   | 291 $\pm$ 31 AB  |
| Kidney T4-ORD  | 195 $\pm$ 42 C   | 235 $\pm$ 39 BC   | 219 $\pm$ 29 BC   | 322 $\pm$ 42 AB   | 408 $\pm$ 75 AB   | 481 $\pm$ 66 A   |

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# REGULATION OF INTERMEDIARY METABOLISM BY THYROID AND INTERRENAL SYSTEMS IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Thyroid and interrenal systems are involved in energy metabolism. Both endocrine pathways produce hormones (T3 and cortisol, respectively) which are considered hyperglycemic, however their effects in fish are still not clear. The aim of this study was to elucidate the relationships of these two systems with the intermediary metabolism of the gilthead sea bream (*Sparus aurata*). For this reason, the following compounds, related to both systems, were administered orally: i) T3, the biologically active thyroid hormone; ii) propylthiouracil (PTU), a thyroid hormone synthesis inhibitor; iii) cortisol, the main corticoid hormone in fish; and iv) dexamethasone, a synthetic glucocorticoid. After 35 days of treatment, various enzymatic activities related to the energy metabolism were analyzed in liver, white muscle and gills. Glycogen phosphorylase (GP), lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH) and 3-hydroxyacyl-CoA dehydrogenase (HOAD) activities pointed to a tight relationship between thyroid and interrenal systems, however several differences can be derived amongst them. The main result is that PTU stimulates lipid catabolism and amino acid turnover, as well as enhances glycogenolysis and anaerobic metabolism in some tissues, while T3 seems to increase only lipid catabolism at hepatic level. On the other hand, cortisol mainly enhances muscular catabolism while dexamethasone increases general catabolism. The differential responses in all the tissues to the compounds tested need further analysis in order to clarify the mechanisms involved in intermediary metabolism and its liaisons with the thyroid and stress axes.

## Introduction

Fish thyroid physiology is complex and involves many different systems (metabolism, growth, osmoregulation, reproduction, amongst others), being different among vertebrates. Under physiological conditions, and regulated by the pituitary-secreted thyroid stimulating hormone, fish thyroid follicles secrete mainly, if not exclusively, thyroxine (T4) (1). This hormone is distributed by blood vessels to the target cells, where it can be deiodinated, producing the biologically active thyroid hormone, triiodothyronine (T3) (2). Thyroid hormones (THs) are involved in the control of many processes such as metabolism (3) or somatic growth (4), among others. Effects of THs on fish metabolism have frequently been studied, but a consistent picture could not be constructed as important differences between species appear to exist (5, 6).

The hypothalamus-pituitary-interrenal system produces corticosteroids (namely cortisol) in fish (7). Cortisol is considered to be the main stress hormone and it

promotes metabolic pathways that increase gluconeogenesis and glycogenolysis, as well as affects amino acid metabolism (8), thus stimulating catabolism of glycogen, lipids and protein (9).

Relationships between thyroid and interrenal axes have been described and seem to share controllers centrally (10). THs and cortisol trigger, alone or combined, the activation or inactivation of several energy metabolic enzymes (11). Anyway, metabolic responses to each hormone seem to vary widely among the different fish species, and no clear pattern of actions could be ascribed (3).

The aim of this study is, therefore, to try to clarify the effects of both axes on the energy metabolism in the teleost species gilthead sea bream (*Sparus aurata*). To achieve this, various compounds related to thyroid and interrenal systems were administered to the specimens, and their effects on several enzymatic activities related to carbohydrate, lipid and protein metabolism on different tissues were analyzed.

## Material and Methods

Juveniles of gilthead sea bream (*S. aurata*) ( $n = 120$ ,  $18.1 \pm 0.2$  g weight and  $9.9 \pm 0.0$  cm length) were distributed randomly in five 400 L tanks. Specimens were maintained in a flow-through system at 40 ppt of salinity, 19 °C and natural photoperiod (fall in the south of Spain). Fish were fed with one of the following compounds: i) control; ii) 10 mg T3 kg food<sup>-1</sup> (11); iii) 5 mg PTU kg food<sup>-1</sup> (Ruiz-Jarabo, data not published); iv) 400 mg Cortisol kg food<sup>-1</sup> (12); and v) 300 mg Dexamethasone (DXM) kg food<sup>-1</sup> (13). Food preparation was conducted as described above (14). After 35 days, 12 animals per group were sampled. White muscle, liver and gills were collected. Glycogen phosphorilase (GP) (15), lactate dehydrogenase (LDH) (16), glutamate dehydrogenase (GDH) (17) and 3-hydroxyacyl-CoA dehydrogenase (HOAD) (modified from 18) activities were analyzed.

## Results and Discussion

Gilthead sea bream juveniles fed with T3, PTU, cortisol or DXM exhibit different energy metabolism effects. GP activity (Table 1) indicates glycogen consumption, and it is increased in the DXM group in every tissue sampled. This enzymatic activity is also stimulated when animals are fed with PTU and cortisol, but only in the muscle. LDH activity (Table 2) is enhanced in all experimental groups in the muscle, while cortisol and DXM stimulated the anaerobic metabolism also in gills. HOAD activity (Table 3) represents lipid catabolism. It is enhanced in the muscle by PTU, cortisol and DXM, in the liver by T3 and DXM, and in the gills by PTU and DXM. GDH activity (Table 4) increased in the muscle and gills when fish are treated with cortisol and DXM, which indicates protein catabolism. This enzymatic activity decreased in the liver of those groups, showing that the amino acids produced by this catabolic process are consumed in the same tissues where they are produced, indicating that the liver is not involved in protein catabolism. On the other hand, PTU increased GDH activity in muscle and gills, which is related to amino acid turnover and protein synthesis.