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Changes in glucose, glycogen, thyroid activity and hypothalamic catecholamines in tench by starvation and refeeding

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Abstract The effects of short-term food deprivation (7 days) and refeeding (2 days) on different biochemical and neuroendocrine parameters were studied in tench. A 7-days fast resulted in a significant reduction of plasma glucose and glycogen hepatic content, supporting the key role of liver glycogen as energy depot for being consumed during fasting. The rapid recovery of normal values of blood glucose and glycogen stores by refeeding indicates a rapid replenishment of liver glycogen stores. The short-term starvation decreased circulating thyroid hormones (both T_3 and T_4) and T_4 release from thyroid, supporting an interaction between nutritional state and thyroid function in tench. All these metabolic and hormonal changes were partial or totally reversed under refeeding conditions. An increase in hypothalamic content of norepinephrine and dopamine was found in fasted fish. This result might be a consequence of stress induced by starvation.

Keywords Carbohydrates · Catecholamines · Fasting · Tench · Thyroid hormones

Abbreviations bw body weight · DA dopamine · DHBA 3,4-dihydroxybenzilamine · E epinephrine · HSI hepatosomatic index · NE norepinephrine · T_3 3',5,3,-triiodothyronine · T_4 thyroxine · TH thyroid hormones · TSH thyroid-stimulating hormone

Introduction

It is known that many fish alternate feeding and fasting periods during the annual cycle as a consequence of reproductive processes or seasonal variations in temperature or food availability (Madrid et al. 2001). A variety of strategies for surviving to different periods of food deprivation have been adopted by fish, including metabolic, hormonal and behavioural responses. As a rule, fish appear to use catabolic energy conservation strategies which meet their caloric needs but minimize their tissue energy loss (Navarro and Gutiérrez 1995). However, the nature of metabolic changes in starvation depends on the species and duration of the fasting period. Certain fish such as goldfish, carp, rainbow trout (Baanante et al. 1991) and porgy (Rueda et al. 1998) preserve glycogen stores while metabolizing lipids and/or proteins. Alternatively, other species, such as cod (Hemre et al. 1993), tilapia (Hsieh and Shiau 2000) and coho salmon (Larsen et al. 2001) conserve protein and lipid while partially depleting glycogen stores.

Several hormones play a central role in regulating nutrient utilization during periods of fasting in all vertebrates, including fish. Thyroid hormones (TH) represent a good candidate signal in the adaptive metabolic response to starvation, and it can exert both hyperglycaemic and glycogenolytic actions. It has been reported that fasting or reduced feeding can down-regulate the hypothalamus-hypophysis-thyroid axis, and whereby, the anabolic activity is inhibited during these periods of starvation (Bentley 1998). In teleost fish, food deprivation reduces thyroid tissue sensitivity to thyroid stimulating hormone (TSH), plasma 3',5,3,-triiodothyronine (T_3) and thyroxine (T_4) concentrations and clearance, liver T_3 nuclear receptors density, and monodeiodinase activity, as well as eliminates plasma TH daily patterns (Cerdá-Reverter et al. 1996; Kühn et al. 1998; Sohn et al. 1998; Van der Geyten et al. 1998; Gaylord et al. 2001).

Brain monoaminergic systems are involved in feeding regulation in vertebrates (De Pedro et al. 1997, 1998; Meguid et al. 2000). Changes in nutrient composition and/or ration of food intake can modify such brain monoamines in homeotherms (Hajnal and Lénárd 1997; Meguid et al. 2000; Tachibana et al. 2000). Some reports have shown the effect of diet on brain monoaminergic

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activity in teleosts (Sloley et al. 1986; Pouliot et al. 1988), but the possible effects of starvation on endogenous brain catecholamines have been scarcely examined.

The tench (*Tinca tinca*) is an economically important cyprinid widely distributed in Europe. Recent studies have shown a seasonal pattern in metabolic resources in this species (Guijarro et al. 2003), clearly influenced by environmental factors such as the food availability. The specific responses of tench to food deprivation and refeeding are unknown. Therefore, the present study was undertaken to examine the effects of 7 days fasting and subsequent access to food for 2 days on hepatosomatic index, plasma glucose levels, liver glycogen content and some neuroendocrine parameters (thyroid hormones in blood and thyroid gland, and hypothalamic content of catecholamines).

Materials and methods

Animals

Tench (6.2 ± 1.4 g body weight, bw) supplied by the "Centro Regional de Acuicultura. Piscifactoría Las Vegas del Guadiana" (Badajoz, Spain), were maintained at the laboratory in glass aquaria (50 l) with painted black walls to avoid stress during the acclimation period. Fish were held in flowing and aerated tap water, under natural photoperiod (15L:9D) and water temperature 21 ± 2°C (Spring) for 1 month before the experiment. Food consisted in Sera Biogran pellets at a daily ration of 1% bw at 10:00 hours.

Experimental procedure

Tench were divided in three groups ($n = 10$/group): (1) fed group (F): fish fed throughout the experimental period with the same daily ration as before (1% bw); (2) starved group (S): 1 week food-deprived animals; and (3) starved and refed group (S + RF): fish were starved 7 days and refed for 2 days (1% bw). Fish were sacrificed by decapitation at 14:00 hours (4 h after the last food in the fed groups). Blood was collected using heparinized capillary tubes and plasma samples were stored at −25°C until analysis. Liver, lower jaws containing the thyroid tissue, and hypothalamus were rapidly removed and frozen on solid CO_2. The tissue samples were stored at −25°C until analysis.

Analytical procedures

Plasma glucose levels were determined by the glucose-oxidase method using a commercial kit (Glucose Trinder, Knickerbrocker Labs). Hepatic glycogen content was measured by spectrophotometry (Dubois et al. 1956) after extraction with ethanol and previous digestion with KOH (Cifonelli et al. 1956; Montgomery 1957).

The extraction procedure of free and bound fractions of thyroid hormones in the thyroid was previously described in detail (De Pedro et al. 1995a, 1995b). Lower jaws containing the thyroid tissue were homogenised in methanol (12 ml g^{-1} wet weight) and centrifuged (4,500 rpm for 15 min). The hormones thus extracted in the supernatant represent the free thyroid T_4 and T_3 contents. Thyroglobulin-bound T_4 and T_3 were obtained after overnight proteolytic digestion of the pellet with pronase (0.58%). Plasma thyroid hormones were extracted and measured as previously described by Morreale et al. (1985) with minor modifications for fish samples (De Pedro et al. 1995b). T_3 and T_4 were extracted with chloroform-methanol (2:1) and $CaCl_2$ (0.05%), and further purified with Bio-Rad AG 1×2 resin columns.

Thyroid hormone content (T_4 and T_3) were determined by highly sensitive and specific RIAs described by Obregón et al. (1979). The limits of detection were 1.5 pg for T_4 and 0.78 pg for T_3 per tube. The intra- and interassay coefficients of variation were 4.16 and 8.55% (1.56 pg, $n = 9$), 7.9 and 18.26% (25 pg, $n = 10$) for T_3; 4.63 and 6.69% (10 pg, $n = 10$), 7.91 and 14.85% (160 pg, $n = 10$) for T_4.

The validation of the RIA for tench samples was performed by comparing the displacement curves obtained with different volumes of either extracted plasma or thyroid samples with standard curves (T_4 and T_3). Parallelism was statistically tested by one-way analysis of variance (ANOVA) between the slopes of standards and sample dilutions, previously calculated by linear regression after logit-log transformation of the data.

Protein content in liver and jaw homogenates was determined by the method of Lowry et al. (1951), using serum bovine albumin as standard.

Hypothalamic content of norepinephrine (NE), epinephrine (E) and dopamine (DA) was quantified by HPLC with coulometric detection (Coulochem II, ESA), as previously described (De Pedro et al. 1997). Hypothalami were homogenised by sonication in 120 μl of cold 0.2 N perchloric acid containing 0.4 mmol l^{-1} sodium bisulphite, 0.4 mmol l^{-1} EDTA and 25 ng ml^{-1} of 3,4-dihydroxybenzilamine (DHBA) as internal standard. The homogenate was centrifuged (13,000 rpm for 1 min) and the supernatant filtered (0.45 mm, Millex-HV13). The mobile phase (flow rate 1 ml min^{-1}) consisted of 10 mmol l^{-1} citric acid, 5 mmol l^{-1} disodium phosphate, 0.05 mmol l^{-1} EDTA, 0.12 mmol l^{-1} sodium octanesulphonic acid and 3% methanol (pH 3). The HPLC system consisted of a Waters 590 pump, a pulse dampener, a Rheodyne injection valve with a 25-μl loop and a C18 reversed-phase column (125 mm×4.6 mm ID, 5 μm particle size). A procedure of oxidation/reduction was used (conditioning cell: +300 mV; analytical cell no. 1: +100 mV; analytical cell no. 2: −250 mV). Signal from analytical cell no. 2 was recorded with a sensitivity of 50 nA on a Waters 746 integrator and results were calculated as the area under peaks and expressed as nanograms per hypothalamus.

Statistical analysis

All data were expressed as mean ± SEM. Data were analysed by ANOVA followed by Duncan's multiple range test for multi-group comparisons. A probability level of $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows parallel displacements of serial dilutions of both plasma and thyroid tissue-extracted samples and the T_3 and T_4 standard curves. There were not significant ($P > 0.05$) differences between the slopes of standards and sample dilutions (Table 1).

Changes in circulating glucose, hepatic glycogen content and hepatosomatic index (HSI) induced by starvation and refeeding in tench are presented in Fig. 2. Plasma glucose levels were significantly ($P < 0.005$) reduced in response to 7 days starvation, which was totally reversed after 2 days refeeding (Fig. 2a). Similarly, significant ($P < 0.005$) reductions in both hepatic content of glycogen and HSI were observed in tench after 7 days starvation (Fig. 2b, c), whereas refeeding for 2 days partially reversed such decreases.

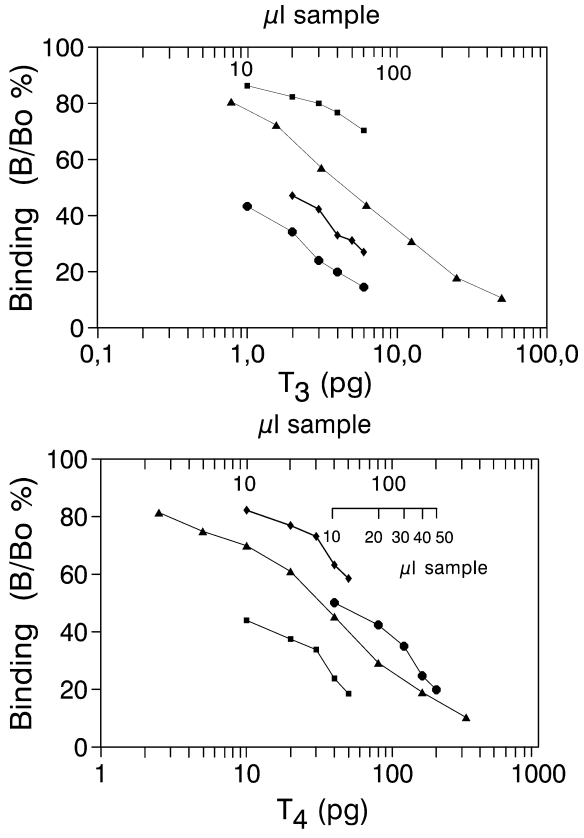

Fig. 1 Comparison of 3',5,3,-triiodothyronine (T_3) and thyroxine (T_4) standard curves (*filled triangles*) with different volumes of thyroid-bound fraction (*filled circles*), thyroid-free fraction (*filled squares*) and plasma (*filled diamonds*) samples. Each point represents the average of duplicate determinations for samples. Standard curves represent the average of three different standard curves. Error bars $< 5\%$

Figure 3 summarises the effects of starvation and refeeding on free and bound T_3 and T_4 thyroid content in tench. No significant changes in thyroid content of free and bound T_3, and bound T_4 after the different feeding conditions were observed. However, starvation significantly ($P < 0.05$) reduced the T_4-free fraction in thyroid tissue, which recovered normal values after 2 days refeeding (Fig. 3b, left). The T_3/T_4 ratio for both free and bound fractions was not statistically modified in any of the studied experimental groups.

Starvation for 7 days induced a significant decrease in both T_3 and T_4 plasma levels ($P < 0.05$ and $P < 0.005$,

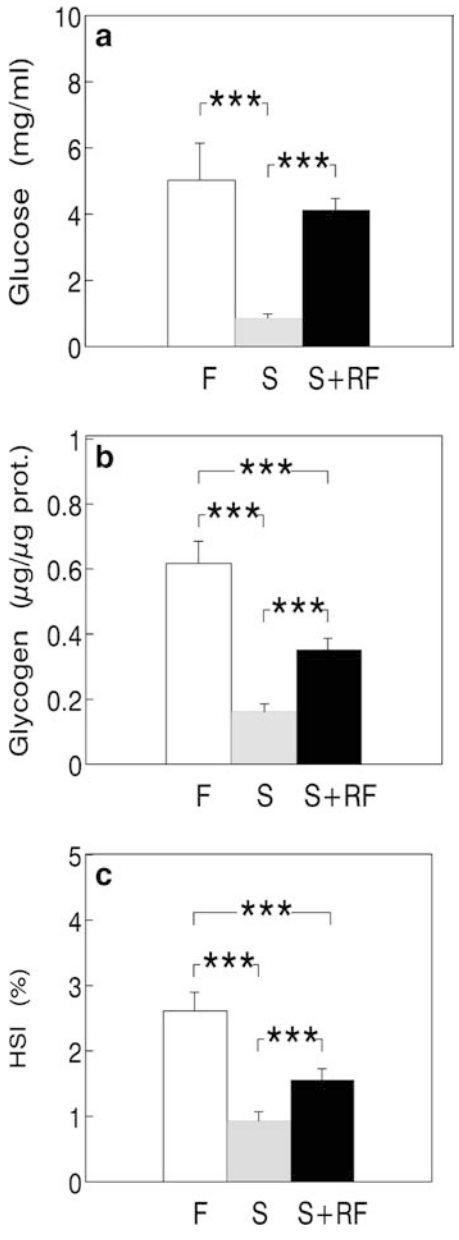

Fig. 2 **a** Plasma glucose levels, **b** hepatic glycogen content and **c** hepatosomatic index (HSI = liver weight/body weight×100) in tench (*Tinca tinca*). *F* feeding (1% body weight, bw) for 7 days; *S* 7 days starvation; *S + RF* 7 days starvation followed by 2 days refeeding (1% bw). Data are expressed as mean ± SEM ($n = 10$/group). ***$P < 0.005$

Table 1 Equations of the regression lines for 3',5,3,-triiodothyronine (T_3) and thyroxine (T_4) standard curves and samples dilution of free and bound fraction and plasma from tench (*Tinca tinca*)

	T_3				T_4			
	Standard curve	Free fraction	Bound fraction	Plasma	Standard curve	Free fraction	Bound fraction	Plasma
A	14.295	12.468	18.297	19.562	12.511	16.144	9.273	10.502
B	3.545	3.266	5.031	4.986	4.721	4.778	4.266	4.067
r	0.983	0.928	0.988	0.983	0.997	0.994	0.99	0.926

A intercept to the *y*-axis; *B* slope of the equations of the regression lines; *r* correlation coefficient

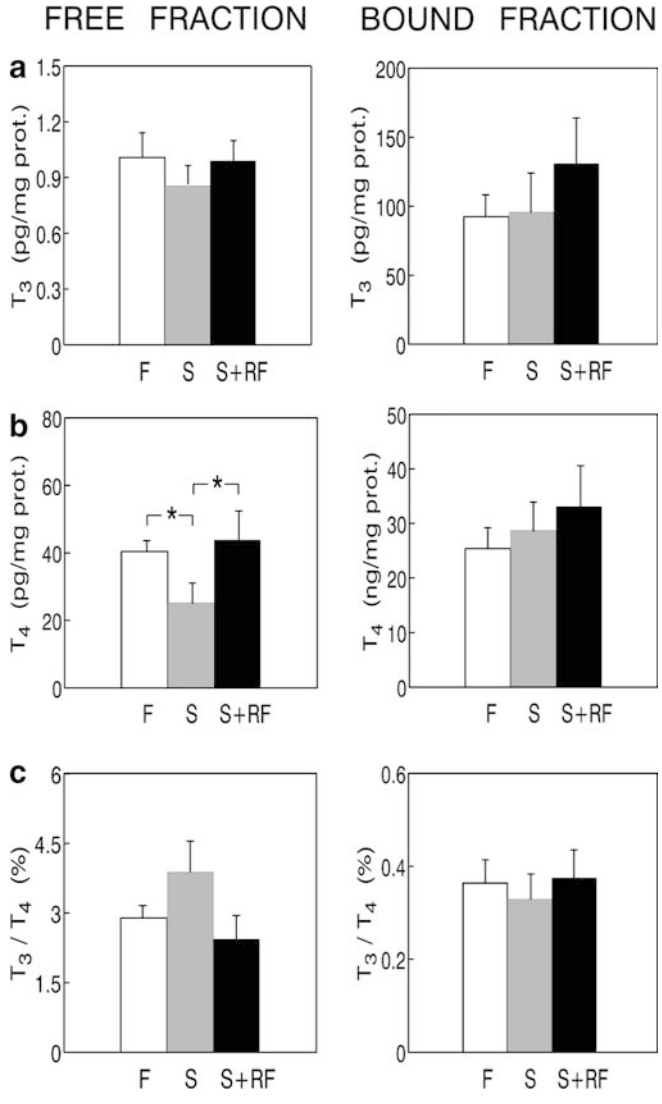

Fig. 3 Thyroid content of free (*left*) and bound (*right*) T_3 (**a**) and T_4 (**b**) fractions, and T_3/T_4 ratio (**c**) in tench (*Tinca tinca*). *F* feeding (1% bw) for 7 days; *S* 7 days starvation; *S+RF* 7 days starvation followed by 2 days refeeding (1% bw). Data are expressed as mean ± SEM ($n = 10$/group). $^*P < 0.05$

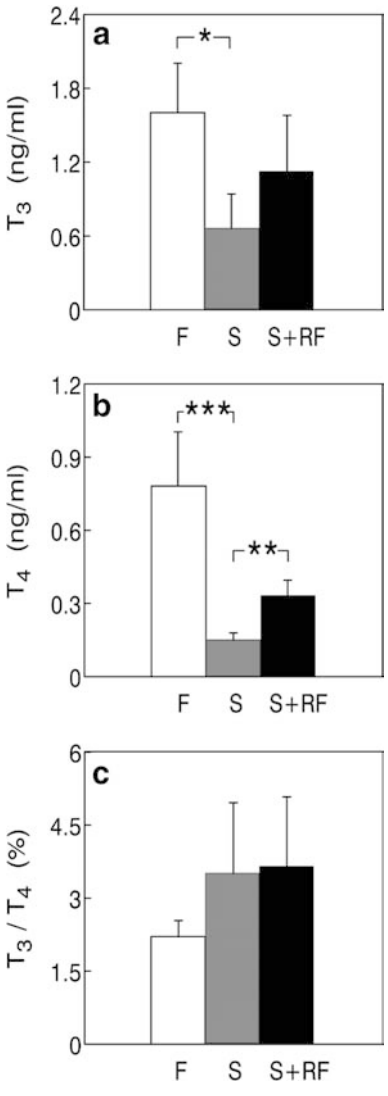

Fig. 4 Plasma levels of **a** T_3, **b** T_4 and **c** T_3/T_4 ratio in tench (*Tinca tinca*). *F* feeding (1% bw) for 7 days; *S* 7 days starvation; *S+RF* 7 days starvation followed by 2 days refeeding (1% bw). Data are expressed as mean ± SEM ($n = 10$/group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$

respectively), that was partially reversed by refeeding for 2 days (Fig. 4). Plasma T_3/T_4 ratio remained unchanged in the different groups of fish.

The hypothalamic content of NE, E and DA after the different feeding paradigms in tench is presented in Fig. 5. Starvation for 7 days significantly increased norepinephrine ($P < 0.01$) and dopamine ($P < 0.005$) content in relation to fed fish (Fig. 5a, c). Refeeding for 2 days did not significantly reverse such effect of fasting. There were no statistically significant modifications of epinephrine hypothalamic content by starvation (Fig. 5b).

Discussion

During early stages of fasting (1 week) tench mobilized liver glycogen depots. This strategy for supplying energy during short starvation periods adopted by tench is in line with other fish species that also use liver glycogen for the maintenance of metabolic functions during early starvation (Hemre et al. 1993; Soengas et al. 1996; Hsie and Shiau 2000, Larsen et al. 2001). Moreover, a fall in plasma glucose levels was clearly produced by fasting in tench. Similar decreases in glycemia have been shown in other species under different periods of food deprivation (Soengas et al. 1996; Figueroa et al. 2000; Blasco et al. 2001). The concomitant decrease in both blood glucose and liver glycogen observed in tench after 7 days starvation indicates that the active glycogenolysis produced to counteract fasting was not enough to maintain the glycemia, which agrees with data obtained in Atlantic salmon (Soengas et al. 1996). However, in starved cod plasma glucose levels are maintained by reducing the rate of glucose utilization and/or increasing

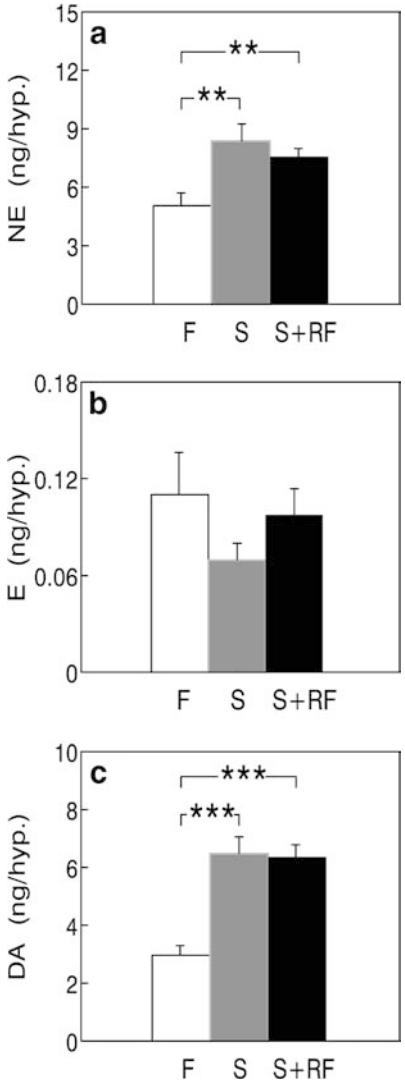

Fig. 5 Hypothalamic content of **a** norepinephrine (*NE*), **b** epinephrine (*E*), and **c** dopamine (*DA*) in tench (*Tinca tinca*). *F* feeding (1% bw) for 7 days; *S* 7 days starvation; *S+RF* 7 days starvation followed by 2 days refeeding (1% bw). Data are expressed as mean ± SEM ($n = 10$/group). $^{**}P < 0.01$, $^{***}P < 0.005$

gluconeogenesis (Hemre et al. 1993). This different response to fasting could be related to many factors, such as interspecies variability, age, past nutritional history, season and others, as it has been suggested (Soengas et al.1996; Boujard et al. 2000).

In the present study, refeeding for 2 days induced a partial recovery of liver weight and glycogen content. This result corresponds with previous studies in teleosts (Böhm et al. 1994; Soengas et al. 1996; Rueda et al. 1998; Larsen et al. 2001) where a rapid restoration of liver reserves during refeeding has been reported, emphasizing the key role played by the liver during short fasting periods. The fact that tench refed for 2 days exhibited normal values of glycemia and significantly recovered hepatic glycogen content indicates that at least a fraction of such increase in post-feeding plasma glucose levels were directed to replenish the exhausted liver glycogen depots, as it occurs in carp (Böhm et al. 1994) and rainbow trout (Figueroa et al. 2000). Nevertheless, the existence of indirect pathways producing glycogenogenesis during refeeding, e.g. from 3-carbon compounds via the gluconeogenic pathway (Baanante et al. 1991), can not be discarded.

Metabolism is controlled by the interaction of many hormones under different nutritional conditions, which keep energy reserves necessary for maintenance of the healthy organism. During periods of fasting or starvation, pancreatic hormones, growth hormone, as well as glucocorticoids play important metabolic roles. However, the possible role of thyroid hormones is unclear at this time, mainly in ectothermic animals. In our study, starvation for 1 week clearly produced an inhibitory effect of thyroid activity in the tench, i.e. a decreased free fraction of intrathyroidal T_4 and circulating thyroid hormones. These results agree with previous reports on the inhibitory action of fasting on different aspects of thyroid function (Cerdá-Reverter et al. 1996; Kühn et al. 1998; Sohn et al. 1998; Van der Geyten et al. 1998; Gaylord et al. 2001).

The fasting-induced reduction of intrathyroidal free T_4 content in tench indicates a decreased secretory activity of the thyroid, bearing in mind that this fraction represents the hormone ready to be released into circulation. Moreover, such hypothetical reduction in thyroidal T_4 secretion is supported by the decrease of TH plasma levels together with an unchanged peripheral deiodination (plasma T_3/T_4 ratio). Nevertheless, there may be interactions with other endocrine systems that are also modified by starvation. Thus, it has been shown that corticosteroids (presumably released during stress situations as starvation) inhibit thyroid function, causing decreases in plasma levels of T_3 and T_4, accompanied or not by a reduced deiodinating activity (Kühn et al. 1998). Moreover, in addition to direct actions of TH on metabolism, these hormones exert a permissive role in the action of other hormones and enzymes involved in the metabolism regulation (Bentley 1998).

Thyroxine is the main thyroidal secretion in many teleost species, being mostly T_3 of extrathyroidal origin (Kühn et al. 1993). Our data support this preferential synthesis and secretion of T_4 by the thyroid in tench, but we have also quantified significant amounts of intrathyroidal T_3, suggesting that this hormone in tench could be released by the thyroid and thus significantly contribute to the plasma T_3 pool. This is in agreement with recent results in sturgeon, which indicate that thyroid can be a significant direct source of T_3 (Plohman et al. 2002). Previous evidences in rainbow trout, where circulating T_3 is more dependent on this thyroidal release than on its formation in peripheral tissues (Sefkow et al. 1996), also corroborate our data in tench.

On the other hand, decreases in circulating levels of TH during the fasting period may point to a general mechanism used by tench to meet a food deprivation period by slowing down the metabolic rate, as suggested

by Hemre et al. (1993) for the cod. The decline in thyroid activity induced by starvation is clearly an adaptive response to reduce metabolism or growth and preserve nutritional reserves. In fact, thyroid hormones could participate in the adjustment of seasonal changes of metabolic activity described for this species (Guijarro et al. 2003), mainly in conditions of limited food supply, as it occurs in winter.

Two days of refeeding allows the recovery of normal values in the metabolic and hormonal parameters studied in tench. Our results suggest a rapid compensatory mechanism of food intake, when tench are returned to full rations of food. There is evidence that periods of food deprivation are followed by hyperphagic responses and compensatory growth (Rueda et al. 1998; Boujard et al. 2000; Gaylord et al. 2001).

The hypothalamic content of catecholamines in tench was similar to that found in others cyprinids, such as goldfish (De Pedro et al. 1997, 1998), but the response of hypothalamic catecholaminergic system to starvation was different depending on species. Thus, fasting induces an increase in hypothalamic NE and DA content in the tench versus a reduction of both catecholamines in goldfish (De Pedro et al. 2001). This discrepancy may result from differences in feeding pattern: tench show nocturnal feeding activity, while goldfish tend to be day active (Sánchez-Vázquez et al. 1996). In contrast to goldfish, tench is highly sensitive to stressful stimuli. Then, it can be hypothesized that the NE and DA increase observed in the present study could be a consequence of starvation-induced stress, as it has been described in starved insects (Barreteau et al. 1993). Recent studies have shown that fish subjected to different stress conditions present an increased serotoninergic and dopaminergic activity (Jobling et al. 1999; Amcoff et al. 2002). The determination of plasma cortisol levels in tench could be relevant to support this hypothesis.

In summary, the present results show for the first time some hormonal and biochemical adjustments adopted by tench to cope with 1 week of fasting, and restoration after 2 days of refeeding. In agreement with findings in other vertebrates, plasma TH levels can also be used as a rapid indicator of nutritional status in tench. Data from the present study represent important reference values useful for aquaculturists and physiologists working with this species.

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