

# STRESS-INDUCED EFFECTS ON FEEDING BEHAVIOR AND GROWTH PERFORMANCE OF THE SEA BASS (*Dicentrarchus labrax*): A SELF-FEEDING APPROACH

Esther Leal<sup>1</sup>, Begoña Fernández<sup>1</sup>, Raul Guillot, Diana Ríos, José Miguel Cerdá-Reverter\*

Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre de la Sal (IATS), Consejo Superior de Investigaciones Científicas (CSIC), Castellón, SPAIN.

**Running Title:** Stress and food intake in fish

**\*Address for Correspondence:** Dr. J.M. Cerdá-Reverter, Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre de la Sal, 12595 Torre de la Sal, Ribera de Cabanes, Castellón, SPAIN. E-mail Address: [cerdarev@iats.csic.es](mailto:cerdarev@iats.csic.es), Phone Number: (+) 34 964319500, Fax: (+) 34 964319509.

<sup>1</sup> These authors contributed equally to this work

## **ABSTRACT**

Repetitive aquaculture-related protocols may act as cyclic stressors that induce chronic stress in cultured fish. The sea bass is particularly sensitive to stressful conditions and the mere presence of humans will disturb feeding behavior. In this paper, we study whether chronic stress induced by repetition of acute stress protocols affects long-term feeding behavior and growth performance in sea bass and whether exogenous cortisol may induce stress-like changes in these parameters. We demonstrate that both chronic stress and dietary cortisol decrease food intake and have a negative effect on feed conversion efficiency, severely impairing sea bass performance. Both experimental approaches induced changes in the daily feeding activity by lengthening the active feeding periods. Fish subjected to a cyclic stressor modify their daily feeding pattern in an attempt to avoid interference with the time of the stressor. The delay in feeding when fish are acutely and repeatedly stressed could be of substantial adaptive importance.

Keywords: Stress, Cortisol, Feeding Behavior, Food Intake, Growth Performance, Food Conversion Efficiency, Daily Rhythms

## INTRODUCTION

The primary stress endocrine response in fish is mainly mediated by the hypothalamic-sympathetic chromaffin cell (HSC) and the hypothalamic-pituitary-interrenal axes (HPI). Both systems contribute in a key manner to restoring homeostasis after stress mainly by mobilizing fuel stores to make energy available for the increased metabolic demand. Sustained reallocation of metabolic energy away from growth processes (both somatic and reproductive) compromises the performance capacity of the fish during chronic stress. Therefore, reduced or negative growth is commonly observed during stressful periods, while growth rates or derived parameters are often considered as reliable indicators of stress and welfare (Wenderlaar-Bonga, 1997; Mommsen et al., 1999; Ashley 2006; Aluru and Vijayan, 2009).

Causes for stress-induced growth retardation are diverse. A reduction in the food intake levels and/or disruption of the feeding behavior is a common feature of the behavioral response to stress in fish (Bernier and Peter, 2001; Bernier 2006). Under stressful conditions fish eat less and grow more slowly than unstressed fish. Stressful conditions are known also to induce reductions in feed conversion efficiency (FCE) that can lead to decreased growth rates even when food intake levels are maintained (Barton et al., 1987; Gregory and Wood 1999; Paspatis et al., 2003; d'Orbcastel et al., 2010). These negative effects of stressors on FCE may be mediated by a disruption of metabolic regulation or increased activity, leading to increased energy expenditure or reducing the absorption of food through the intestine (Barton et al., 1987; Mommsen et al., 1999). In addition, stressors can modify the regulation of the endocrine growth axis including pituitary growth hormone (GH) secretion and hepatic insulin-like growth factors (IGFs) synthesis (Rotllant et al., 2001; Dean and Woo; 2009; Saera-Vila et al., 2009).

Cortisol is the main glucocorticoid in fish and the end-product of the HPI axis activation (Wenderlaar-Bonga, 1997). Elevation of corticosteroid plasma levels is one of the most

evolutionary conserved stress responses and it is commonly used as an indicator of the degree of stress experienced. Cortisol is thought to mediate many effects of stressors on physiological, metabolic and behavioral processes (Wenderlaar-Bonga, 1997; Mommsen et al., 1999; Barton, 2002; Aluru and Vijayan, 2009). Evidence suggests that cortisol is the main mediator of stress-induced growth suppression since chronically elevated plasma levels following its exogenous administration reduce fish growth by simulating stress effects, i.e. reduction in food intake levels and FCE, increased energy expenditure and compromised food absorption (Barton et al., 1987; Gregory and Wood, 1999; De Boeck et al., 2001).

The sea bass (*Dicentrarchus labrax*), is an important species in Mediterranean and Atlantic aquaculture and a number of studies have focused on its feeding behavior (Sánchez-Vázquez et al., 1995a,b; Boujard et al., 1996; Sánchez-Vázquez et al., 1998; Azzaydi et al., 2007; Leal et al., 2009). Under ambient conditions, feeding patterns display a marked seasonality. Grouped animals are diurnal during the summer-autumn but exhibit nocturnal feeding during the winter (Sánchez-Vázquez et al., 1998). However, individually reared fish exhibit a dual pattern of daily feeding, some fish displaying diurnal behavior while others are strictly nocturnal under identical culture conditions. Animals spontaneously invert the phasing of their daily feeding pattern (diurnal fish become nocturnal and vice versa) but the mechanisms involved are unknown (Sánchez-Vázquez et al., 1995a,b). Sea bass is very sensitive to stressful conditions and the mere presence of humans can significantly reduce feeding activity (Rubio et al., 2010). Rearing density severely affects sea bass performance (Paspatis et al., 2003; Roque d'Orbcastel et al., 2010) as well as the response to acute stress challenges (Di Marco et al., 2008). Cortisol has been suggested to mediate density-induced effects on sea bass growth but the extent and manner of its involvement remain unexplored. This investigation follows our previous work in the sea bass, in which we observed that acute stress challenges can modify short-term food intake (Rubio et al., 2010). In this study, we investigate: 1) whether chronic stress, different from rearing density, can affect long-term feeding behavior and

growth performance and 2) whether exogenous cortisol may induce stress-like changes in these parameters. To this end, we first investigated the effects of the stress induced by routine tank cleaning practices on daily food intake and feeding pattern, specific growth rate (SGR) and FCE. In a second experiment, we evaluated the effect of a 30 day feeding trial with cortisol-containing diets on the same feeding and biometric parameters. The results demonstrate that both chronic stress and dietary cortisol can depress feeding, growth and FCE, and modify daily feeding rhythms in the sea bass.

## **MATERIAL AND METHODS**

### **Animals**

One year old immature sea bass [body weight (BW)=  $120.9 \pm 0.87$  g and length (L)=  $20.79 \pm 0.06$  cm] were maintained in 2000 L tanks supplied with continuously aerated running sea water and equipped with automatic feeder activated by a string sensor placed 3 cm below the water surface. The feeders were connected to a computer system that recorded the date, the time and the tank from which each food demand originated. The number of demands was integrated every 5 minutes. Animals were maintained under natural conditions for six months and self-fed with a commercial diet (Mistral 21, Proaqua Nutrición, S.A.; 43% protein, 23% fat, 20% carbohydrates, 6% ash, gross energy 22.5 kJ/g, in 3 mm standard pellets). Before the experiments, fish were placed in the experimental 500 L tanks, continuously supplied with running seawater and provided with identical self-feeding systems, and acclimated for at least one week. The full water volume of the tank was renovated every 40 minutes. The experimental tanks were visually isolated from the remaining tanks in the culture facilities so that routine activities did not disturb the fish. No access to the experimental area was allowed, except for sampling and cleaning procedures. Prior to netting, animals were preanaesthetized in 2-phenoxy-ethanol (0.02%) for 3-5 minutes in their home tanks.

Subsequently, animals were removed from their home tanks and anaesthetized for 2 min in the same anesthetic (0.1%) in the sampling tank. The day before samplings, sensors were removed from the water at 10.00 am, the time at which sampling always started . When required the experimental animals were sacrificed by rapid decapitation. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals.

### **Hormone administration**

The amount of cortisol (hydrocortisone; Sigma St. Louis, MO) to reach the experimental doses (0, 50, 200 and 500 µg/g food) were dissolved in 50 ml ethanol (100%). The solution was sprayed onto 500 g food and dried overnight (O/N) at room temperature (RT) and stored at 4° C until needed. Cortisol-containing food was then administered by hand (experiment 2) or loaded into the feeder containers, and changed every week during the experimental period (experiment 3).

### **Experimental procedure**

***Experiment 1.*** The first trial was a pilot experiment designed to evaluate the short-term effects of physical stress on cortisol plasma levels in the sea bass. Sixty animals (BW= 106.98 ± 1.53 g and L= 20.87 ± 0.102 cm) were distributed into 6 experimental tanks and hand-fed at 2 % BW for two weeks at 9.00 am. After the accommodation period, three tanks were cleaned whereas the animals in control tanks remain undisturbed. The cleaning protocol was always performed at 10.00 am and involved draining and brushing the tanks with the animals inside. The tanks were emptied until the dorsal fins of the fish were exposed and then brushed for 2 minutes and immediately refilled. Blood samples were obtained at 2 and 8 h post-stressor. Animals were not fed during the experimental day.

*Experiment 2.* The second trial was designed to evaluate the effects of repetitive physical stress on sea bass growth performance and feeding behavior. One hundred animals (BW=  $221.82 \pm 1.32$  g L=  $25.95 \pm 0.047$  cm) were distributed into 10 experimental tanks (n=10) provided with automatic self-feeders. Three tanks were cleaned once a week (Monday, 1W) and three tanks were cleaned three times a week (Monday, Wednesday and Friday, 3W), whereas the four control tanks were never cleaned (CTRL). The cleaning protocol was as before. At the beginning of the experiment, one animal from each tank was sacrificed to obtain biometric parameters and blood samples. At the end of the experiment, nine animals/treatment were sampled to obtain the same biometric parameters and blood samples. Food demands were registered during thirty two consecutive days and 10.00 am was considered the beginning of a new 24-h period. The feed reward per sensor activation was set at 0.9 g/demand, which approximately corresponds to 9 food pellets/activation and, by extension, 1 pellet/fish/demand. The total amount of food distributed was calculated by weighing the food remaining in the food hoppers. This quantity was used to calculate the delivery rate for each electronic feeder. The daily food intake was calculated using the feeder delivery rate and the number of daily demands.

*Experiment 3.* The third trial was a pilot experiment to evaluate the effect of cortisol administration in the fish diet. Twenty one animals of approximately 150 g were fed at 2 % BW for two weeks with control food at 10.00 am. Subsequently, seven animals were fed with the control diet, while 14 fish were given the hormone-containing food at 50  $\mu\text{g/g}$  (n=7) or 500  $\mu\text{g/g}$  food (n=7) at the same time of day for 10 days. On the sampling day, animals were fed at 10.00 am and two hours later blood samples were obtained by caudal puncture. The plasma was stored at -20 °C until assayed.

*Experiment 4.* The fourth trial was designed to evaluate the effects of cortisol administration on sea bass growth performance and feeding behavior. Ninety animals (BW=  $136 \pm 0.96$  g L=  $22.63 \pm 0.05$

cm) were distributed into 9 experimental tanks (n=10) provided with automatic self-feeders. Three tanks were fed the control diet (CTRL), three tanks the cortisol-containing food at 200 µg/g food (C200) and the remaining three tanks with cortisol-enriched diet at 500 µg/g food (C500). At the beginning of the experiment, one animal from each tank was sacrificed to obtain biometric parameters and blood samples. At the end of the experiment, 9 animals/treatment were sampled for the same purpose. All the fish were weighed and measured. The daily food intake was calculated as above (see experiment 1).

### **Hormone analysis**

Plasma cortisol levels were measured by ELISA (Neogen Corporation) according to the manufacturer's instructions. Dilution curves showed that cortisol rabbit antisera identified the cortisol present in both standard solutions and intact plasma in a similar manner (data not shown).

### **Result expression and statistical analysis**

Data concerning food intake, biometric parameters and plasma hormone levels are expressed as means ± standard error. Specific growth rates were calculated as  $g(X) = 100 * [(\ln X_F - \ln X_0) / t]$ .  $X_F$  and  $X_0$  indicate the value of the variable [body weight (BW), length (L), hepatosomatic index (HSI), mesenteric fat index (MFI) or condition factor (CF)] at the end ( $F$ ) and beginning ( $0$ ) of the experiment, respectively. Condition factor was calculated as  $BW (g) / L^3 (cm)$ . HSI were calculated as  $100 X [liver weight (g) / BW (g)]$ . MFI was calculated as  $100 X [weight of the fat around the viscera (g) / BW (g)]$ . FCE was calculated as  $total food intake / (BW_F - BW_0)$ . B indicates biomass. Differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test ( $P < 0.05$ ). For feeding rhythm studies, food intake level values were integrated per hour and represented as percentage of the total food intake. Daily variations were fitted by means of the cosine function with circadian periodicity:  $Y = M + A [(\cos (\Omega * t + \Phi))]$ , where  $M =$



mesor (mean level of the variable calculated throughout a whole cycle,  $A$  = amplitude,  $\Omega$ =angular frequency,  $t$ =time and  $\Phi$ = acrophase; fits were considered significant when  $p < 0.01$ ).

## RESULTS

*Experiment 1.* Acute physical stress imposed by cleaning induced a significant increase of cortisol plasma levels 2 h after the stressor ceased. Significant differences were not detected at 8 hours post-treatment (Fig.1).

*Experiment 2. Effect of repetitive physical stress on sea bass feeding behavior and growth performance*

Acute physical stress imposed by cleaning once or three times a week significantly reduced daily and cumulative food intake levels (Figure 2A; 3A) as well as FCE (Table 1). Control fish were heavier but not longer and, accordingly, exhibited a higher CF than that shown by fish stressed once a week (Table 1). All three growth rates; i.e.  $g(BW)$ ,  $g(L)$ ,  $g(CF)$  were higher in control fish but only  $g(BW)$  and  $g(L)$  reached significant differences (Table 1). Similarly HSI and MFI values were higher in control fish than treated fish but differences did not reach statistical relevance. At the end of the experiment, no differences in plasma cortisol levels were observed (Table 1).

The temporal ingestive pattern of fish was similar in all treatments and demands were mainly recorded during the light phase of the photoperiod (Fig. 4A). All three treatments showed significant variations in food intake levels during the 24-h cycle which fitted significantly circadian cosine curves ( $t=24$  h, data not shown). Control fish exhibited a significant increase in food intake between 8 and 10h, when they consumed approximately 28% of their daily feed intake. During this time interval, stressed fish, on the other hand, ate approximately 10% of the total daily feed. Fish stressed three times a week ate significantly more than control and 1W fish between 17 and

19h. No significant differences in the amplitude or acrophase of the rhythms were detected (data not shown). Control and 1W fish demanded more than mesor levels (4.16 %) from 9 to 15h, 3W fish demanded more from 10 to 15h.

### *Experiment 3. Exogenous cortisol administration*

The administration of dietary cortisol at highest dose significantly increased plasma cortisol levels two hours after feeding (Fig. 5). On the contrary, the lowest dose tested resulted in no significant increase. Overall plasma cortisol in fish fed the highest doses were 5.8 times higher than cortisol levels in control fish ( $167 \pm 15.3$  ng/ml). Therefore, we decided to feed the animals with dietary cortisol at doses of 0 (CTRL), 200 (C200) and 500 (C500)  $\mu\text{g/g}$  food.

### *Experiment 4. Effects of exogenous cortisol on sea bass feeding behavior and growth performance*

The administration of the dietary cortisol induced a significant decrease in the daily food intake levels, which led to a dose-response reduction in the cumulative food intake (Figs 2B; 3B). FCE was also significantly reduced in fish fed the hormone-containing diet (Table 2). Differences in food intake and FCE resulted in heavier and longer fish in the control group than in those treated with cortisol (Table 2). Accordingly, fish treated with the steroid exhibited lower specific growth rates expressed as weight [(g(BW))], length [(g(L)] or CF [(g(CF))] than the control fish (Table 2). In fact, CF increased in the control fish, whereas treated fish lost condition (Table 2). Accordingly, control fish preserved their mesenteric fat percentage, whereas this parameter significantly decreased in treated fish. A similar tendency was observed in the HSI but differences did not reach statistical significance (Table 2).

All three treatments showed led to significant variations in food intake levels during 24-h cycles which displayed significant fits to circadian cosine curves ( $t=24$  h). Fish ate mainly during the light

phase of the photoperiod and acrophases were fitted around midday. However cortisol-treated fish showed a significant food intake peak at 20.00-21.00h, when they consumed approximately 12% of the total daily feed (Fig.4B). Five out of six cortisol-treated groups displayed the same pattern while only one fish group of the C500 treatment follow a similar pattern to that exhibited by all the three control groups, which never ate more than 2% of the total daily food intake during this period. This discrepancy was responsible for the absence of significant differences during this period in the food intake level of the C500 fish compared with the control group (Fig.4B). This peak did not give rise to significant differences in the amplitude or acrophase (data not shown) but extended the period of active feeding in cortisol-treated fish (Fig.4B). Therefore, control fish showed a higher number of demands than the mesor levels (4.16 %) from 9 to 18h, whereas treated fish demanded more than mesor levels from 8 to 21h.

## **DISCUSSION**

Our previous results demonstrated that acute stress arising from human activity close to tanks, cleaning or sampling protocols have a profound effect on sea bass food intake although fish exhibit a compensatory response once the stressor has ceased (Rubio et al., 2010). Our present results add to these findings by showing that if such stressful conditions become chronic, fish cannot compensate, and daily and accumulated food intake, FCE and growth rates are severely depressed. This explains why the specific growth rates were 10.2 % and 17.2 % for  $g_{BW}$  and 23.0 and 38.4 % for  $g_L$  lower in animals stressed once or three times a week than in control fish, respectively. Similarly, MFI and CF were reduced by 17.5 and 14.1% and 4.4% and 2.2%, respectively. This reduction in the percentage of body fat and condition suggest increased energy expenditure in stressed animals although it might also result from a combined reduction in food intake and FCE levels. The 1W and 3W stressed fish did not exhibit significant differences in daily or accumulated

food intake levels and growth performance, indicating the absence of a graded response to the frequency of the stressor and suggesting the susceptibility of the sea bass performance to chronic stressors. This finding differs from those previously reported for sea bass in which repetitive acute stressors did not induce behavioral or performance changes (Millot et al., 2010). Results on the effects of chronic stress induced by rearing density on sea bass performance are controversial. Stock density up to 45 kg/m<sup>3</sup> did not affect the energy status of sea bass and their sensitivity to subsequent acute crowding stressor (Di Marco et al., 2008). Reduced daily food intake and specific growth rate were observed at densities of 100 kg/m<sup>3</sup> but FCE was unaltered (Roque d'Orbcastel et al., 2010; Sammouth et al., 2009). However, experiments with similar sized animals demonstrated negative effects on FCE at 50 kg/m<sup>3</sup>, whereas the growth performance was reduced at 75 kg/m<sup>3</sup> (Santos et al., 2010). Overall, the data indicate that chronic stress induced by high stock densities have negative effects on sea bass performance, supporting our results obtained using chronic physical stressors.

Significant differences in the accumulated food intake levels of stressed fish were observed at 11 days and were maintained until the end of the experiment. The differences in accumulated food intake in the stressed fish increased from day 11 to the end of the experiment, suggesting that fish did not exhibit any compensatory response driven by a stress adaptation mechanism. As expected during the summer period, all fish groups exhibited a diurnal feeding pattern with acrophases at around midday (Azzaydi et al., 2007; Rubio et al., 2010). The feeding pattern of control animals slightly differed from those observed in stressed fish. Control fish exhibited higher food demand activity during the first hours of the light phase of the photoperiod, while fish stressed 3W demanded significantly more during the last hours of the light phase. Experiments in sea bass have reported that the use of combined physical stressors repeated randomly does not modify the daily feeding patterns (Millot et al., 2010). However, differences in feeding rhythms of the sea bass mediated by chronic stress induced by stocking density have been reported. With crowding, sea bass

tend to lengthen the feeding activity period surpassing the nocturnal restriction (Paspatis et al., 2003). Repeated acute stressors are thought to alter behavior mainly by reducing feeding activity during the stress period which is associated to decreased growth rates (Millot et al., 2010 and references therein). In our experiment, the stress protocol always was applied at 10.00 am so fish might have postponed their maximal demands until after the stressor had terminated. In some way, fish seemed to learn the time when the stressor would take place. The delay in feeding behavior when fish are acutely and repeatedly stressed could be of substantial adaptive importance.

Most stress-induced effects on growth performance and behavior are mediated by increased plasma cortisol levels (Wendeerlar-Bonga, 1997; Mommsen et al., 1999; Aluru and Vijayan, 2009). Consequently, we evaluated the cortisol levels in the experimental fish but no significant differences were observed at the end of the experiment. In a previous pilot experiment, we demonstrated that the cleaning protocol induce a significant increase in plasma cortisol levels after two hours but differences were resumed into the following 6 hours. Similar results were obtained in sea bass subjected to an acute crowding stressor (Di Marco et al., 2008) although when crowding stressor persist for 24h, plasma cortisol levels remained significantly upraised (Rotllant et al. 2003). Animals of the experiment 2 were sampled at 48 h post-stressor. Therefore, it is conceivable that no differences in plasma cortisol levels were found.

In the following experiment we tested whether the administration of exogenous cortisol could simulate the results obtained in the previous experiment. A second pilot experiment was designed to corroborate that the dietary cortisol is able to increase plasma levels as previously reported in the goldfish (Bernier et al. 2004). Animals fed with the highest dose of dietary cortisol (C500) displayed a significant increases in plasma cortisol levels which were similar to those previously reported in crowded or acute stressed sea bass (Simontacchi et al., 2008; Lupatsch et al., 2010). However, lower tested doses (C50) did not induce significant differences compared with control animals. Therefore, we decided to use an intermediate dose (C200) to observe any graded response

in the plasma cortisol levels. This study is the first to examine the effects of chronic cortisol increase on daily food intake and growth performance using self-feeding systems. It cannot be affirmed unequivocally that cortisol added to the surface of the feed does not affect the taste of the feed. However, to the best of our knowledge, cortisol does not stimulate (or otherwise) the gustatory system of fish. Studies in goldfish suggested that dietary cortisol does not affect the gustatory response (Bernier et al., 2004). In addition, differences in food intake levels were obtained after 18 days of treatment and one could expect suppressant or deterrent substances to induce a faster taste response in food intake levels (Kasumyan and Doving, 2003). Animals treated with the highest cortisol dose (C500) lost weight, displaying a negative specific growth rate. Similarly, g<sub>BW</sub> in animals treated with the lowest cortisol dose (C200) was 77.6 % lower than in control fish. The condition factor of the control animals increased throughout the experiment but the cortisol-treated animals lost body condition, probably as partial result of a severe decrease in the mesenteric fat content. Therefore, the decreased (C200) or suspended (C500) growth in cortisol-treated fish was probably a combined result of decreased food intake and efficiency levels and even increased energy expenditure as suggested by the severe reduction in body fat levels. Evidence in other fish species suggests that cortisol administration suppresses growth by increasing energy expenditure through the stimulation of energy-demanding metabolic processes such as lipid mobilization (Mommsen et al., 1999; De Boeck et al., 2001; Bernier et al., 2004). Cortisol also can interfere with the hormonal system involved in the regulation of growth (Kajimura et al., 2003) but more experiments are needed to reveal its involvement in the regulation of the sea bass GH/IGF axis. The results obtained for growth performance are in good agreement with those obtained previously in other fish species. Experiments in rainbow trout demonstrated that cortisol implantation dramatically reduced food intake levels, leading to depressed g(BW) and FCE (Gregory and Wood, 1999). Similar results were reported in goldfish treated with high cortisol doses, while lower doses stimulated food intake without promoting growth (Bernier et al., 2004). Our experimental design

cannot confirm that the observed effects are specifically mediated by glucocorticoid receptors, but the time elapsing between the treatment and the response suggests that classical glucocorticoid receptors working through genomic actions are involved in the observed response. Sea bass glucocorticoid receptor has been cloned and hepatic expression is downregulated in crowded sea bass exhibiting higher cortisol levels than control animals (Terova et al. 2005).

Feeding behavior and, by extension, food intake levels are regulated in the central nervous system, where the neuronal hypothalamic circuits integrate incoming visceral and sensorial information to orchestrate an integrated feeding response. The inferior hypothalamic lobe, particularly areas close to the lateral recess, as well as the ventro-posterior hypothalamus, are involved in the control of feeding behavior (reviewed by Volkoff et al., 2005). There are no data concerning the central expression of glucocorticoid receptor in sea bass but studies in rainbow trout showed that this receptor is profusely expressed within the ventral telencephalon and preoptic area, as well as in the tuberal and hypothalamic lobes (Teistma et al., 1998). Central anorexigenic effector pathways downstream of glucocorticoid receptors in fish remain to be established. Studies in goldfish demonstrated that peripheral cortisol administration inhibits corticotropin releasing factor (CRF) but stimulates neuropeptide Y (NPY) expression within the preoptic area (Bernier et al., 2004). This scenario may explain the orexigenic effects of cortisol doses since NPY and CRF are known to stimulate and inhibit, respectively, food intake in goldfish (Volkoff et al., 2005) and mammalian species (Cavagnini et al., 2000). Experiments in rainbow trout have demonstrated that implanted cortisol inhibits preoptic expression of both CRF and NPY but does not prevent the stimulation of neuropeptide expression under stress, suggesting that the responsiveness to acute stressors is maintained under chronic stress (Doyon et al., 2006). Overall, the data suggest that the fish neural system involved in the control of food intake is responsive to increased plasma cortisol levels but that factors other than NPY and CRF mediate the anorexic effects of cortisol in a more significant manner. However, more experiments are needed to identify such central factors.

The administration of dietary cortisol seemed to simulate the stress-induced effects as the treated fish exhibited a dose-response reduction in accumulated food intake and severe effects on growth performance. However, the timing and magnitude of the changes were different. The differences in food intake were obtained at 11 and 18 days post-treatment in physically stressed and cortisol-treated fish, respectively. Cortisol-induced effects on growth rates and FCE were much more pronounced than those induced by chronic physical stress. In addition, cortisol treatment, but not chronic physical disturbance, had a severe effect on g(CF) and g(MFI). Such discrepancy in the magnitude of the response could be explained in terms of differential intensity of the stimulus. In chronically stressed fish, the negative stimulus was only present once or three times a week but hormone-treated fish received exogenous cortisol during the whole experiment. Therefore, a more severe response could be expected in cortisol-treated fish. Differences in the timing of the response are more difficult to explain since stress effects on food intake were reached earlier than the hormone-induced effects. However, stress effects are not mediated entirely by increased plasma cortisol levels which represent the final outcome of the HPI axis activation. The upstream activation of the axis can mediate also the activation and/or inhibition of different metabolic and behavioral pathways. Our results have demonstrated that ACTH induce hepatic lipolysis via activation of the melanocortin 2 receptor (Agulleiro MJ, Sánchez E, Fernández B, Leal E, Guillot R and Cerdá-Reverter unpublished results). On the other hand, increased CRF levels during stress response could activate different neuronal pathways involved in the control of food intake than those regulated by the circulating cortisol. In addition, the stress response is not only mediated by HPI axis activation since the HSC axis plays a key role in the fish stress response (Wenderlar-Bonga, 1999). It is conceivable that the cortisol-induced effects on HSC system are different from those induced by stress.

As in the repetitive physical stress experiment, sea bass exhibited a diurnal feeding rhythm with acrophases around midday. Cortisol administration modified the food demand rhythms in the sea



bass, since treated fish exhibited a significant food-demand peak during the last period of the photophase, which was absent in the control fish. This increased feeding activity caused an extension of the demand period in treated fish towards the scotophase. Feeding pattern in the sea bass is seasonal. Grouped animals prefer feed during photophase in summer and during scotophase in winter (Azzaydi et al., 2007). However, when individually reared some fish exhibit diurnal behavior while others are nocturnal. Animals can invert the phasing of the daily feeding pattern, but the mechanisms involved are unknown (Sánchez-Vázquez et al., 1995a,b). Evidence obtained in our experiments are insufficient to confirm the involvement of cortisol and/or stress in the regulation of temporal feeding patterns in the sea bass and more experiments are required to elucidate the effects of stress on the temporal organization of feeding.

In conclusion, chronic stress induced by repetition of acute stress protocols and dietary cortisol administration induced a significant attenuation of food intake and FCE, severely impairing sea bass performance. In addition, both stress protocols and cortisol treatments induced modification of the feeding activity rhythms, suggesting involvement of the stress response in the temporal organization of feeding behavior. Finally, our study demonstrates the sensitivity of sea bass performance to aquaculture-related stressors since only one tank cleaning process in the week was sufficient to severely impair food intake, conversion and growth.

## **ACKNOWLEDGEMENTS**

This work was supported by grants AGL2007-65744-C03-02, AGL2010-22247-C03-01 and CSD 2007-00002 from Spanish Science and Education Ministry (MEC) to JMC-R. Additional funding was obtained from the “Generalitat Valenciana” (research grant PROMETEO 2010/006). EL was recipient of a Geronimo Forteza fellowship from the Generalitat Valenciana (GV). The authors wish to acknowledge TINAMENOR for supplying the experimental animals. We also thank Matias Sánchez for his technical assistance in fish maintenance and cleaning protocols.



## REFERENCES

- Aluru N, Vijayan MM (2009) Stress transcriptomics in fish: a role for genomic cortisol signaling. *Gen Comp Endocrinol* 164: 142-150.
- Ashley PJ (2007) Fish welfare: Current issues in aquaculture. *Appl Anim Behav Sci* 104: 199-235.
- Azzaydi M, Rubio VC, Martínez López FJ, Sánchez-Vázquez FJ, Zamora S, Madrid JA (2007) Effect of time-restricted feeding on annual rhythms of demand-feeding and food anticipatory activity in European sea bass. *Chronobiol Internat* 24: 859-874.
- Barton BA, Schreck CB, Barton LD (1987) Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow-trout. *Dis Aquat Org* 2: 173-185.
- Bernier NJ (2006). The corticotrophin-releasing factor system as mediator of the appetite-suppressing effects of stress in fish. *Gen Comp Endocrinol* 146: 45-55.
- Bernier NJ, Bedard N, Peter RE (2004). Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. *Gen. Comp. Endocrinol.* 135, 230-240.
- Bernier NJ, Peter RE (2001) The hypothalamic-pituitary-interrenal axis and the control of food intake in teleost fish. *Comp Biochem Physiol* 129: 639-644.
- Boujard T, Jourdan M, Kentouri M, Divanach P (1996) Diel feeding activity and the effect of time-restricted self-feeding on growth and feed conversion in European sea bass. *Aquaculture* 139: 117-127.
- Cavagnini F, Croci M, Putignano P, Petroni ML, Invitti C (2000) Glucocorticoids and neuroendocrine function. *Int J Obes Relat Metab Disord* 2, S77-9.

- De Boeck G, Alsop D, Wood C (2001) Cortisol effects on aerobic and anaerobic metabolism, nitrogen excretion, and whole-body composition in juvenile rainbow trout. *Physiol Biochem Zool* 74: 858-868.
- Dean EE, Woo NYS (2009). Modulation of fish growth hormone levels by salinity temperature, pollutants and aquaculture related stress: a review. *Rev Fish Biol Fisheries* 19: 97-120.
- Di Marco P, Priori A, Finoia MG, Massari A, Mandich A, Marino G (2008) Physiological responses of European sea bass *Dicentrarchus labrax* to different stocking densities and acute stress challenge. *Aquaculture* 275: 319-328.
- d'Orbcastel ER, Lemarie G, Breuil G, Petochi T, Marino G, Triplet S, Dutto G, Fivelstad S, Coeurdacier JL (2010) Effects of rearing density on sea bass (*Dicentrarchus labrax*) biological performance, blood parameters and disease resistance in a flow through system. *Aquat Liv Res* 23: 109-117.
- Doyon C, Leclair J, Trudeau VL, Moon TW (2006) Corticotropin-releasing factor and neuropeptide Y mRNA levels are modified by glucocorticoids in rainbow trout, *Oncorhynchus mykiss*. *Gen Comp Endocrinol* 146: 126-135.
- Gregory TR, Wood CM (1999) The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. *Physiol Biochem Zool* 72: 286-295.
- Kajimura S, Hirano T, Visitacion N, Moriyama S, Aida K, Grau EG (2003) Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus*. *J Endocrinol* 178: 91-99.
- Kasumyan AO, Doving KB (2003) Taste preferences in fishes. *Fish and Fishseries* 4: 289-347.
- Leal E, Sánchez E, Muriach B, Cerdá-Reverter JM (2009) Sex steroid-induced inhibition of food intake in sea bass (*Dicentrarchus labrax*). *J Comp Physiol B*. 179: 77-86.

- Lupatsch I, Santos GA, Schrama JW, Verreth JAJ (2010) Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax*. *Aquaculture* 298: 245-250.
- Millot S, Pean S, Leguay D, Vergnet A, Chatain B, Begout ML (2010) Evaluation of behavioral changes induced by a first step of domestication or selection for growth in the European sea bass (*Dicentrarchus labrax*): A self-feeding approach under repeated acute stress. *Aquaculture* 306: 211-217.
- Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fish* 9: 211-268
- Paspatis M, Boujard T, Maragoudaki D, Blanchard G, Kentouri M (2003) Do stocking density and feed reward level affect growth and feeding of self-fed juvenile European sea bass? *Aquaculture* 216: 103-113
- Rotllant J, Balm PH, 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.
- Rubio VC, Sánchez E, Cerdá-Reverter JM (2010) Compensatory feeding in the sea bass after fasting and physical stress. *Aquaculture* 298: 332-337.
- Saera-Vila A, Caldach-Giner JA, Prunet P, Pérez-Sánchez J (2009) Dynamics of liver GH/IGF axis and selected stress markers in juvenile gilthead sea bream (*Sparus aurata*) exposed to acute confinement: differential stress response of growth hormone receptors. *Comp Biochem Physiol A* 154: 197-203.
- Sammouth S, d'Orbcastel ER, Gasset E, Lemarie G, Breuil G, Marino G, Coeurdacier JL, Fivelstad S, Blancheton JP (2009) The effect of density on sea bass (*Dicentrarchus labrax*) performance in a tank-based recirculating system. *Aquacult Engineering* 40: 72-78.

- Sánchez-Vázquez FJ, Zamora S, Madrid JA (1995a) Light-dark and food restriction cycles in sea bass - effect of conflicting zeitgebers on demand-feeding rhythms. *Physiol Behav* 58: 705-714.
- Sánchez-Vázquez FJ, Azzaydi M, Martínez FJ, Zamora S, Madrid JA (1998) Annual rhythms of demand-feeding activity in sea bass: evidence of a seasonal phase inversion of the diel feeding pattern. *Chronobiol. Internat.* 15: 607-622.
- Sánchez-Vázquez FJ, Madrid JA, Zamora S (1995b). Circadian rhythms of feeding activity in sea bass, *Dicentrarchus labrax* L: Dual phasing capacity of diel demand-feeding pattern. *J Biol Rhythm* 10: 256-266.
- Santos GA, Schrama JW, Mamauag REP, Rombout JHWM, Verreth JAJ (2010) Chronic stress impairs performance, energy metabolism and welfare indicators in European sea bass (*Dicentrarchus labrax*): The combined effects of fish crowding and water quality deterioration. *Aquaculture* 299: 73-80.
- Simontacchi C, Poltronieri C, Carraro C, Bertotto D, Xiccato G, Trocino A, Radaelli G (2008) Alternative stress indicators in sea bass *Dicentrarchus labrax*, L. *J Fish Biol* 72: 747-752.
- Teitsma CA, Anglade I, Toutirais G, Muñoz-Cueto JA, Saligaut D, Ducouret B, Kah O (1998) Immunohistochemical localization of glucocorticoid receptors in the forebrain of the rainbow trout (*Oncorhynchus mykiss*). *J Comp Neurol* 401: 395-410.
- Terova G, Gornati R, Rimoldi S, Bernardini G, Saroglia M (2005) Quantification of a glucocorticoid receptor in sea bass (*Dicentrarchus labrax*, L.) reared at high stocking density. *Gene* 357: 144-151.
- Volkoff H, Canosa LF, Unniappan S, Cerdá-Reverter JM, Bernier NJ, Kelly SP, Peter RE (2005) Neuropeptides and the control of food intake in fish. *Gen Comp Endocrinol* 142: 3-19.
- Wenderlar Bonga S (1997) The stress response in fish. *Physiol Rev* 77: 591-625.

## FIGURE LEGENDS

### Figure 1

Plasma cortisol levels after physical disturbance imposed by cleaning protocols with animals inside the tanks. Control fish were undisturbed. The cleaning protocol was always performed at 10.00 am and involved draining and brushing the tanks with the animals inside. Asterisk indicates significant differences between control and disturbed fish after ANOVA followed by Tukey's multiple range test ( $P<0.05$ )

### Figure 2

(A) Cumulative food intake level after repetitive physical disturbance imposed by routine cleaning protocols with animals inside the tanks once (1W) or three times a week (3W). Control fish (CTRL) were never disturbed. Differences were detected 11 days after the beginning of the experiment. (B) Cumulative food intake level after dietary cortisol administration at 0 (CTRL), 200 (C200) or 500 (C500)  $\mu\text{g/g}$  food. Differences were obtained 18 days after the beginning of the experiment. Each point represents the mean  $\pm$  SEM of food intake levels of at least 3 tanks containing nine animals. Asterisk indicates significant differences between control and treated fish after ANOVA followed by Tukey's multiple range test ( $P<0.05$ ),  $\delta$  denotes significant differences from CTRL and C200 treatments.

### Figure 3

Daily mean food intake after repetitive physical disturbance (A) or cortisol treatment (B). Each bar represents the mean + SEM of 132 (CTRL and 1W) or 99 (3W) determinations and 96 determination each for CTRL, C200 and C500. Different letters indicate significant differences after ANOVA followed by Tukey's multiple range test ( $P<0.05$ ). See Figure 1 for more details.

#### **Figure 4**

Daily food intake rhythms in sea bass subjected to repetitive physical disturbance (A) or fed cortisol-containing diets (B). Values are the average of food demanded every 60 minutes expressed as percentage of the total food intake for at least 3 groups of 9 fish each. Black and white areas at the bottom of the graphs represent the dark and light phases, respectively, of the photoperiod. See Figure 1 for more details.

#### **Figure 5**

Plasma cortisol levels of sea bass fed with cortisol-containing diets at doses of 0 (CTRL), 50 (C50) and 500 (C500)  $\mu\text{g/g}$  food fed for 10 days. Animals were hand fed every day at 10.00 h and sampled two hours after food administration (12.00 h). Each bar represents the mean + SEM of 7 determinations. Asterisk indicates significant differences between control and treated fish after ANOVA followed by Tukey's multiple range test ( $P < 0.05$ ).



Table 1. Effects of repetitive physical stress on sea bass growth performance

<b>Treatment</b>	<b>CTRL</b>	<b>1W</b>	<b>3W</b>
<b>BW (g)</b>	269,214±,48 <b>a</b>	250,76±2,95 <b>b</b>	254,38±5,37 <b>b</b>
<b>L (cm)</b>	27,00±0,13	26,77±0,12	26,72±0,13
<b>CF (%)</b>	1,36±0,01 <b>a</b>	1,30±0,01 <b>b</b>	1,33±0,02 <b>ab</b>
<b>HSI (%)</b>	1,80±0,06	1,74±0,07	1,55±0,08
<b>MFI (%)</b>	5,87±0,91	4,85±0,44	5,05±0,40
<b>g(BW) (%)</b>	0.579±0,016 <b>a</b>	0,397±0,055 <b>b</b>	0,405±0,058 <b>b</b>
<b>g(L) (%)</b>	0,126±0,005 <b>a</b>	0,098±0,009 <b>b</b>	0,081±0,001 <b>b</b>
<b>g(CF) (%)</b>	0,205±0,022	0,101±0,038	0,157±0,029
<b>gHSI (%)</b>	-0.76	-0.86	-1.22
<b>gFVI (%)</b>	-1.86	-2.46	-2.33
<b>FCE</b>	2,01±0,02 <b>a</b>	2,56±0,03 <b>b</b>	2,53 ±0,23 <b>b</b>
<b>Cortisol (ng/ml)</b>	150.3±16.38	97.5±12.01	121.4±14.91

1W and 3W denote tanks cleaned once (Monday) and three times a week (Monday, Wednesday and Friday), respectively. Control tanks were never cleaned (CTRL). Data are expressed as means ± standard error. Specific growth rates were calculated as  $g(X) = 100 * [(\ln X_F - \ln X_0) / t]$ .  $X_F$  and  $X_0$  indicate the value of the variable [body weight (BW), length (L), condition factor (CF) hepatosomatic index (HSI) and mesenteric fatty index (MFI)] at the end ( $F$ ) and beginning ( $0$ ) of the experiment, respectively. Feed conversion efficiency (FCE) was calculated as total food intake / ( $B_F - B_0$ ). B indicates biomass. Different letters in the same column indicate significant differences after ANOVA followed by Tukey's multiple range test ( $P < 0.05$ ).

**Table 2. Effects of cortisol administration on sea bass growth performance**

<b>Treatment</b>	<b>CTRL</b>	<b>C200</b>	<b>C500</b>
<b>BW (g)</b>	179,23±2,89 <b>a</b>	143,26±2,56 <b>b</b>	131,78±2,56 <b>b</b>
<b>L (cm)</b>	24,31±0,03 <b>a</b>	23,23±0,14 <b>b</b>	22,92±0,11 <b>b</b>
<b>CF (%)</b>	1,24±0,02 <b>a</b>	1,14±0,01 <b>ab</b>	1,09±0,03 <b>b</b>
<b>HSI (%)</b>	1,33±0,07	1,28±0,08	1,17±0,11 <b>b</b>
<b>MFI (%)</b>	5,67±0,01 <b>a</b>	4,20±0,07 <b>b</b>	3,74±0,19 <b>b</b>
<b>g(BW) (%)</b>	0,85±0,05 <b>a</b>	0,19±0,03 <b>b</b>	-0,1±0,08 <b>b</b>
<b>g(L) (%)</b>	0,23±0,01 <b>a</b>	0,10±0,01 <b>b</b>	0,04±0,01 <b>b</b>
<b>g(CF) (%)</b>	0,16±0,07 <b>a</b>	-0,11±0,03 <b>ab</b>	-0,23±0,05 <b>b</b>
<b>gHSI (%)</b>	-0,78±0,18	-0,88±0,19	-1,19±0,28
<b>gFVI (%)</b>	-0,07±0,05 <b>a</b>	-1,01±0,05 <b>b</b>	-1,38±0,15 <b>b</b>
<b>FCE</b>	1,63±0,13 <b>a</b>	6,39±1,55 <b>b</b>	-5,31±2,51 <b>b</b>

C200 and C500 indicates fish fed with diets containing 200 and 500 µg/g food, respectively. CTRL denotes control fish

See Table 1 for more details.

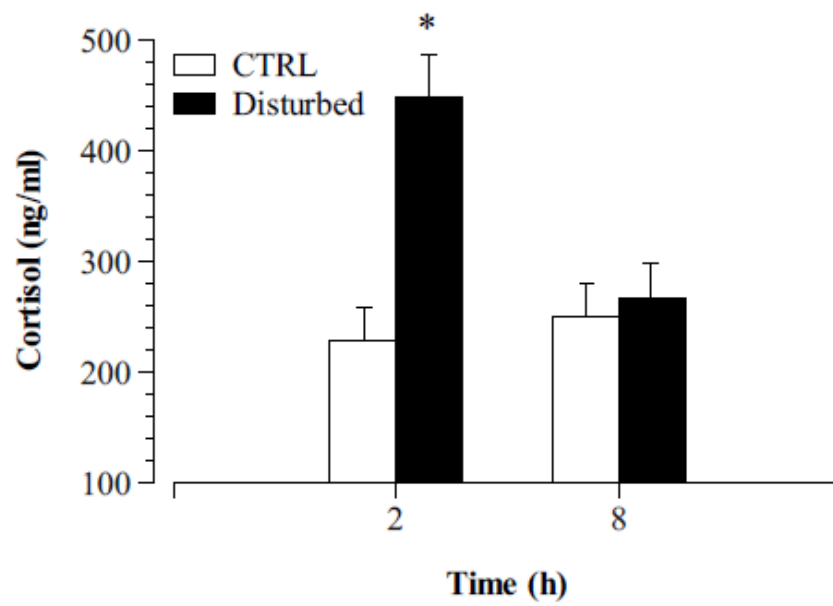


Figure 1

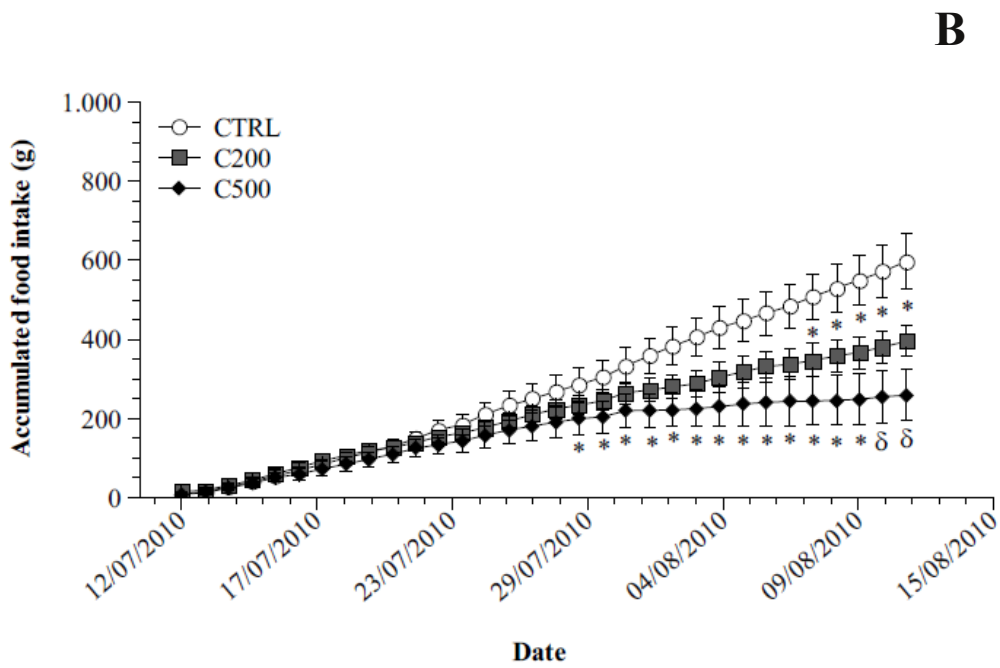
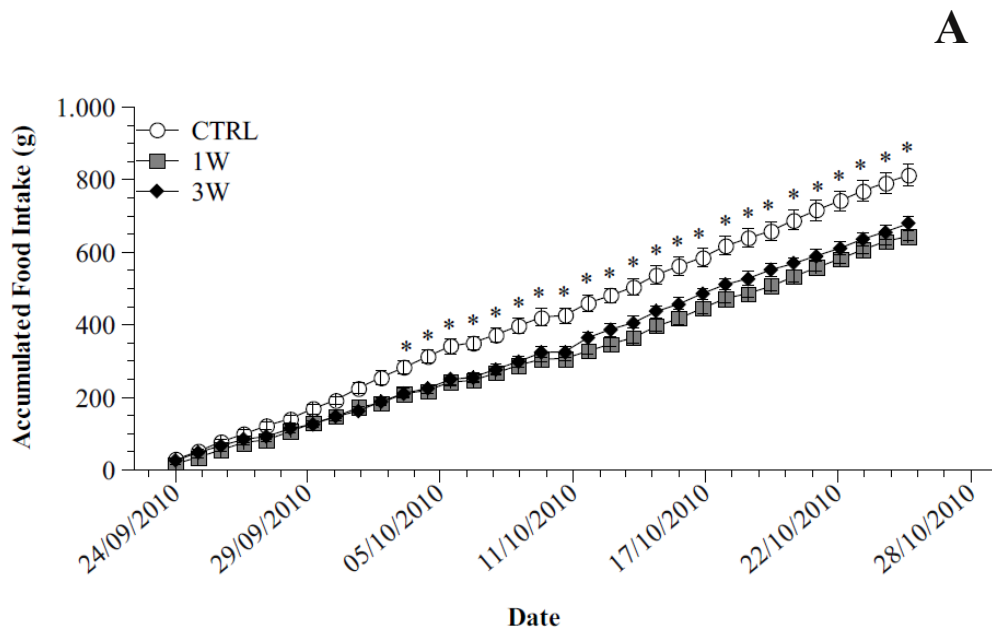


Figure 2

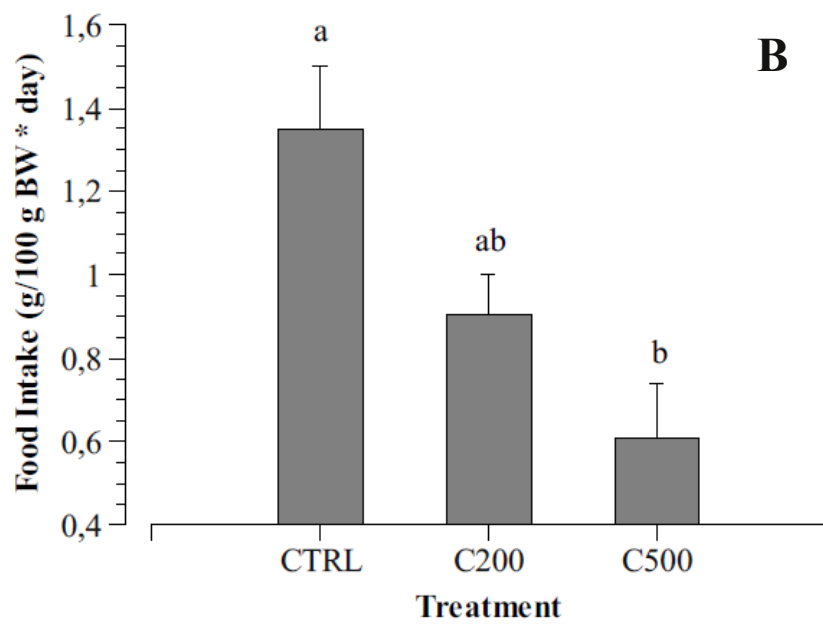
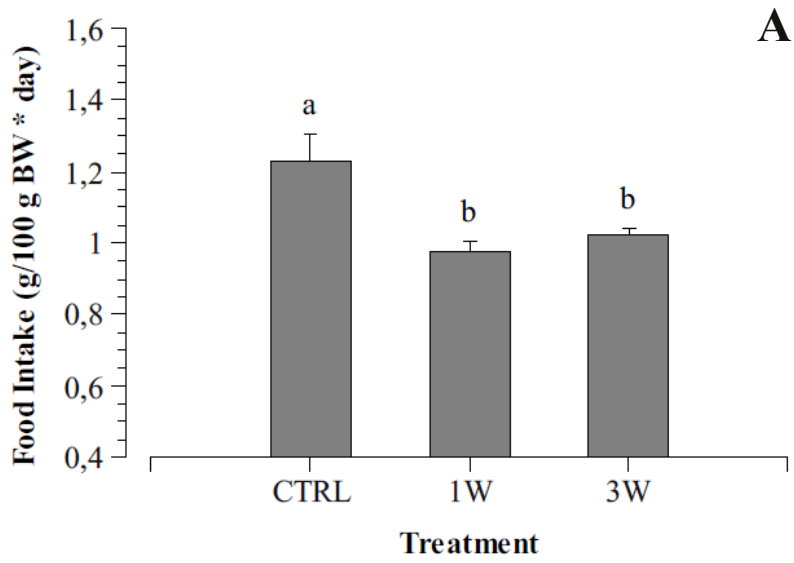


Figure 3

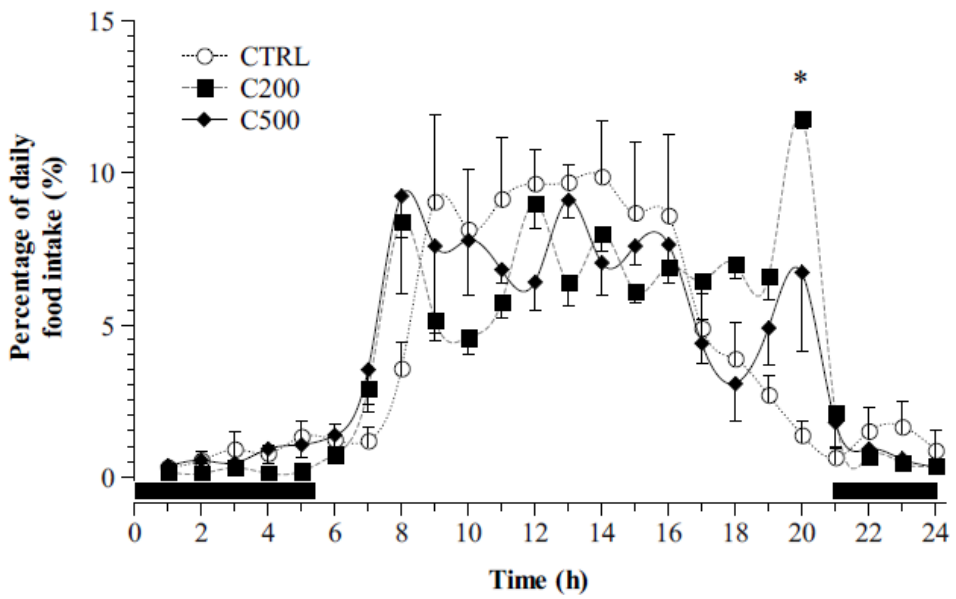
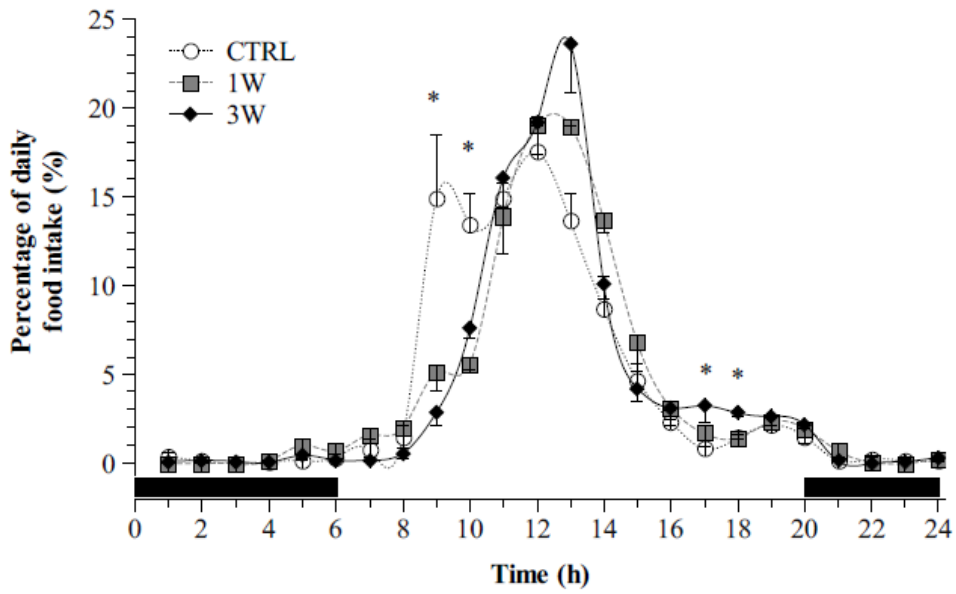


Figure 4

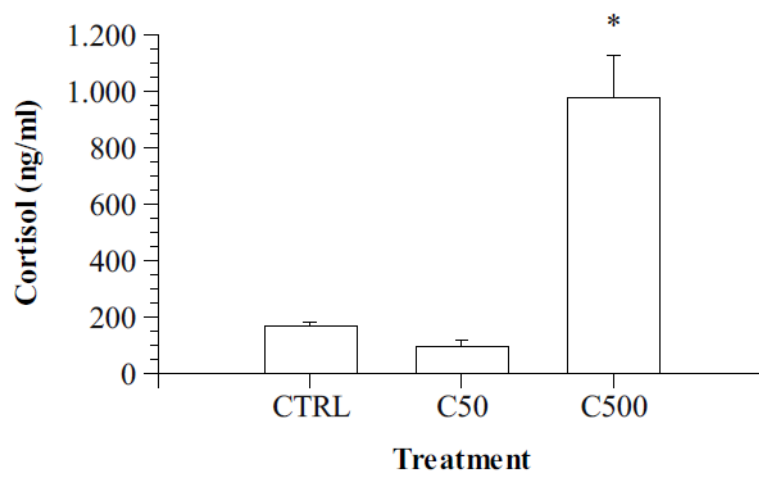


Figure 5