Haematological and Immunological Responses in Juvenile Sea Bass (*Dicentrarchus labrax* L.) After Short-Term Acute Stress

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Abstract: The physiological effects of acute stress (induced by an intraperitoneal injection) on the haematocrit, haemoglobin, serum cortisol and glucose, haemagglutinins and lysozyme were investigated in juvenile sea bass *Dicentrarchus labrax*. A total of 40 fish were subjected to an intraperitoneal injection. Eight fish were sampled at 0, 2, 4, 24 and 48 h after acute stress and compared to unstressed fish (controls) sacrificed at the same times. Intraperitoneal injection resulted in significant changes in cortisol and glucose 4 and 2 hours after exposure to stress, respectively, but not in haematocrit and haemoglobin levels. No significant differences between stressed and control fish were recorded for lysozyme content and haemagglutinating activity throughout the experiment. In juvenile sea bass acute stress caused by intraperitoneal injection was mostly associated with the increase in common stress indicators (cortisol and glucose), while no clear response in terms of non-specific immune parameters was evidenced.

Key Words: *Dicentrarchus labrax*, acute stress, intraperitoneal injection, cortisol, glucose, haematocrit, haemoglobin, non-specific immune parameters.

INTRODUCTION

Many husbandry activities in intensive aquaculture, like handling, transport and vaccination, lead to stress in reared organisms which, if intense or chronic, can eventually lead to fish welfare impairment.

It is well known that high welfare of farmed fish means a good production [1], as proved in many other terrestrial animals. Then research on the effects of aquaculture procedures on welfare is extremely important to produce data and recommendations for best practice and future legislation.

As in other vertebrates, fish experiencing stress show a number of physiological changes; therefore it is important to understand the physiological response to stress response in fish.

In Teleosts stressors elicit a typical "stress response" of adaptive value [2] that is characterized by an immediate release of stress hormones followed by a series of biochemical and physiological changes [3].

In particular fish react to stress with a primary neuroendocrine response, represented by a rapid hypersecretion of catecholamines (adrenaline and noradrenaline) and corticosteroids (mainly cortisol) into the blood stream [4].

As a result of their high levels in the circulatory system, a wide range of secondary responses can be observed; in particular secondary responses are defined as the subsequent actions and effects of these hormones at blood and tissue level, and include disturbance of the metabolic and hydromineral balance.

Tertiary responses include behavioural modifications, implications for fish growth and reproduction and increased sussceptibility to diseases [5,6].

A variety of biochemical measurements is used as indicators of stress in fish.

Among the most frequently measured variables, there are levels of circulating corticosteroid hormones (mainly cortisol) and glucose, lactate, haemoglobin, proteins and haematocrit [1]. In addition, some components of innate immune system (e.g. lysozyme, haemolytic and haemagglutinating activity) are used as indicators of immunocompetence in fish exposed to stress [7-10].

There is extensive literature on the physiological responses of fish to a wide variety of acute and chronic stressors [2, 11-15].

Chronic stressors are usually associated with reduced growth, changes in reproductive function and behaviour (changes in colour, breathing frequency, social and swimming behaviour) [5] and increased susceptibility to disease [16].

On the contrary acute stressors can have different effect in fish; if stress is severe enough, it can have lethal consequences [17, 18] but can also lead to an enhancement of fish immune response (increased concentrations of specific plasma protein, such as lysozyme or complement) and to a better protection against any possible damage [19, 20].

The magnitude and duration of biochemical stress response can vary between species [6, 21, 22].

Most research on the acute stress has been focused on handling [6, 19, 23, 24] and capture [25-28] but no information is available on physiological effects of vaccination.

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Indeed vaccination, especially when practised *via* intraperitoneal (considered the most effective method for delivering vaccines to fish) could cause side-effects and animal welfare impairing.

The purpose of the present study was to simulate intraperitoneal vaccination in order to investigate possible physiological side-effects of this practice (a typical short-term acute stress) in juvenile sea bass *Dicentrarchus labrax* (Linneo, 1758) evaluating changes occurring in some physiological parameters.

In particular the panel of assayed parameters included measures of haemoglobin and haematocrit values, serum glucose and cortisol levels, haemagglutinating activity and the content of lysozyme in plasma, kidney and mucus.

MATERIALS AND METHODOLOGY

Experimental Design and Samples Collection

Juveniles of sea bass (*D. labrax*) were obtained from a commercial Sicilian fish farm.

The study was carried out at the facilities of the Institute for Coastal Marine Environment of Messina.

A group of 80 fish (mean body weight 10.0 g) were randomly distributed in 10 tanks of 100-L volume (eight fish for tank, to avoid the stress repeated capture) and maintained for 2 weeks to allow acclimation.

During this adaptation period fish were fed with a commercial pellets for carnivorous fish administered until satiation.

Each tank was supplied with a constant aerated flow of seawater. The water temperature was 24-25 °C, pH was 8.2 and dissolved oxygen was 7-8 mg L^{-1}. Photoperiod was kept natural.

Before stressor application fish were starved for 18 h. Fasting before fish handling is a common practice in aquaculture especially when using anaesthesia; indeed it avoids the risk of regurgitation which can obstruct the gills and dirty water; normally fasting for at least 24 hours is recommended, in this study we limited fasting to 18 hours. After this period, fish from 5 tanks were anesthetized in MS-222 (tricaine methamesulfonate, 0.1gL^{-1}, Sigma-Aldrich), subjected to an intraperitoneal injection (IP) (without injecting any substance) and then put again in their home tanks. A control group was left undisturbed under the same rearing conditions as the stressed group.

At predetermined time intervals (0, 2, 4, 24 and 48 h post stress) eight stressed fish from each tank were quickly netted, killed by spiking method (destruction of the brain by a sharp spike) and sampled.

Fish were sampled at the same time of each day (at 10.00 am) since the parameters measured (especially cortisol and glucose) are subject to diurnal variations.

Sampling periods were selected on the basis of literature concerning the acute stress response [29], where physiological effects of acute stress are short-term effects that usually occur especially in the first hours after disturbance (up to 24-48 h).

Simultaneously, eight unstressed fish were sacrificed at the same time intervals to be used as controls.

To avoid a sampling-induced stress response the time between dipnet introduction and withdrawal lasted less than 3-4 minutes for group (withdrawal was carried out simultaneously by two operators).

Blood samples were drawn separately from the caudal vein of each individual, in order to monitor haematological and non-specific immune defence parameters. Small volumes of blood, collected in heparinised (14 International Units mL^{-1}) tubes, were used for the immediate determination of haematocrit and haemoglobin values. The remaining fraction was centrifuged and the obtained plasma was stored at - 80°C for further lysozyme assay.

In order to extract the serum for the determination of cortisol, glucose and haemagglutinins, blood samples not treated with heparin, were allowed to clot at 4°C, centrifuged at 1500g for 10 minute and stored at - 80°C until analysis.

From each fish mucus and kidney samples were also collected for lysozyme measures.

Haematological, Biochemical and Non-Specific Immune Response Parameters

Serum cortisol concentration was determined by use of a commercially available enzyme-linked (ELISA) immunoassay kit (Alpha Diagnostic International, USA).

Serum glucose level was determined by a commercial kit based on the reaction of GOD-POD (Glucose Oxidase-Peroxidase) (Sclavo Diagnostics, Italy).

Haematocrit value (% red blood cell) was determined in heparinised capillary tubes after centrifugation in a standard microhaematocrit centrifuge at 12.000 g for 10 minute and comparison of the capillary tube with a reference scale.

Blood haemoglobin concentration was measured colorimetrically by use a cyanmethemoglobin method-based kit (Sclavo Diagnostics, Italy).

Aliquots of plasma, mucus and kidney were assayed for lysozyme content. The assay was performed using the radial diffusion method in agarose plates containing 1% agar added with 0.05% lyophilised *Micrococcus lysodeikticus* (Sigma-Aldrich, Italy) as the substrate, dissolved in pH 5.75 phosphate buffer. Lysozyme content was evaluated by measuring the diameter of lysis produced after incubation at 30°C for 22 h, which was converted into Units of lysozyme per mL of sample (U mL^{-1}) through calibration with known amounts of egg-white lysozyme (Sigma-Aldrich) used as the standard [30, 31].

The haemagglutinating activity of the serum was determined in 96-well microtiter U plates (NUNC INC., Denmark) according to the twofold serial dilution procedure. After addition with a 2% (v/v) sheep erythrocyte suspension in PBS, serial dilutions of the serum were incubated at 35°C for 1 h and at + 4°C overnight; results were reported as log2 value of the final serum dilution showing visible agglutination.

Statistical Analysis

All results are expressed as means ± SE. Data were subjected to two-way analysis of variance (ANOVA) to assess the effects of single variables (groups, sampling times) and of their interaction on physiological parameters. In the design of the statistical analysis groups (control and IP) and sampling times (hours) represented the two main factors. When a significant interaction was found, single comparison between data were performed using one-factor ANOVA. Differences were considered statistically significant when $p \leq 0.05$.

RESULTS

No mortality was observed throughout the experiment.

The effects of stress on haematological and immunological parameters are shown in Figs. (**1-8**).

Fish subjected to stress did not differ significantly from controls in either haematocrit or haemoglobin concentration (Figs. **1-2**).

Cortisol levels (ng mL^{-1}) ranged from 184.13 to 381.82 in control fish and from 181.76 to 620.45 in stressed fish.

Significant elevation in cortisol levels was observed in fish that experienced acute stress.

Serum cortisol markedly increases 2 hours after disturbance, and the peak was detected after 4 hours when cortisol reaches a maximum concentration of 620.45 ng mL^{-1}. This value was significantly ($p< 0.01$) higher than control group.

Subsequently, serum cortisol value decreased but, after 48 h, it remained significantly higher than in the control group ($p < 0.05$) (Fig. **3**).

Serum glucose (mg dL^{-1}) ranged from 52.39 to 101.06 and from 52.40 to 121.40 in control and stressed fish, respectively.

Unlike cortisol, serum glucose, in stressed fish, raised significantly after 2 hours, reaching a maximum value of 121.40 mg dL^{-1}. This value was significantly higher than control group ($p<0.01$).

After two hours glycaemia rapidly decreased and concentration remained significantly ($p< 0.01$) lower than control group, up to the end of the trial (48 h post stress) (Fig. **4**).

Lysozyme content varied differently depending on the tissue or organ where it was measured (Figs. **5-7**).

On average, the lowest values were recorded in the plasma of the control group, whose lysozyme values ranged from 1.11 to 1.24 U mL^{-1}, compared to the stressed group whose values increased, ranging from 0.99 to 1.48 U mL^{-1}.

Although not statistically significant by ANOVA, the highest difference in the lysozyme values between the two groups was found 4 hours after intraperitoneal vaccination (Fig. **5**).

In the mucus of the control group lysozyme content ranged from 1.48 to 1.79 U mL^{-1}, while in the stressed one lower lysozyme values were measured, ranging from 1.48 to 1.73 U mL^{-1}. No significant differences between the two groups were recorded throughout the experiment (Fig. **6**).

In the kidney lysozyme values ranged from 1.24 to 2.22 U mL^{-1} in the control group, whereas in the stressed one values were lower, ranging from 1.11 to 1.73 U mL^{-1}; a slight increase in lysozyme levels was recorded 4 hours after stress (Fig. **7**).

The haemagglutinating activity was generally lower in the stressed fish, where log2 values ranged from 3 to 4, compared to the values recorded in the control fish, ranging from 4 to 5. The activity slightly increased 4 hours after stress, although a similar trend was observed in control group; afterward; stressed fish maintained values similar to those measured in control ones (Fig. **8**).

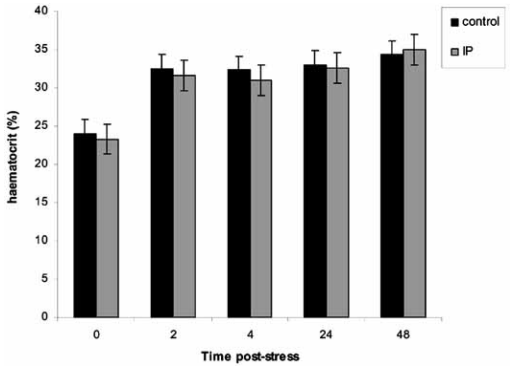

Fig. (1). Haematocrit value in control and stressed (IP) juvenile sea bass. Values are means ± SEM (n = 8).

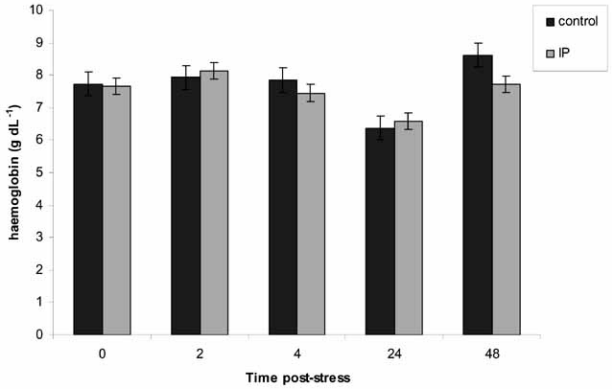

Fig. (2). Haemoglobin concentration in control and stressed (IP) juvenile sea bream. Values are means ± SEM (n = 8).

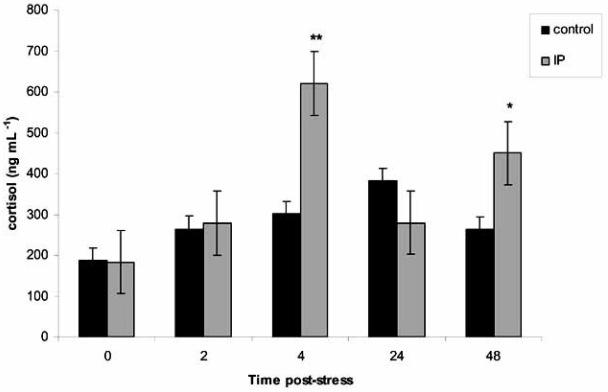

Fig. (3). Cortisol concentration in control and stressed (IP) juvenile sea bass. Values are means ± SEM (n = 8). * Significantly different from the control *($P \leq 0.05$) **($P \leq 0.01$).

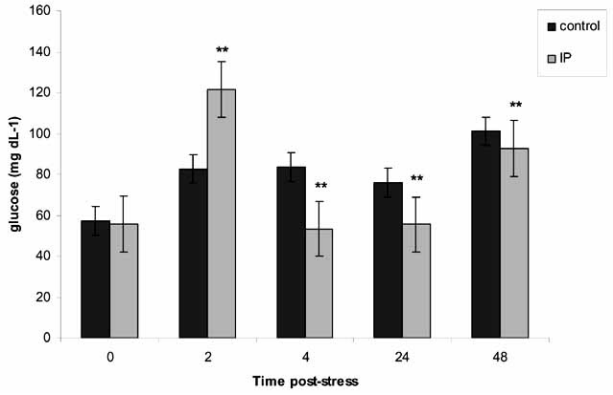

Fig. (4). Glucose concentration in control and stressed (IP) juvenile sea bass. Values are means ± SEM (n = 8). * Significantly different from the control **($P \leq 0.01$).

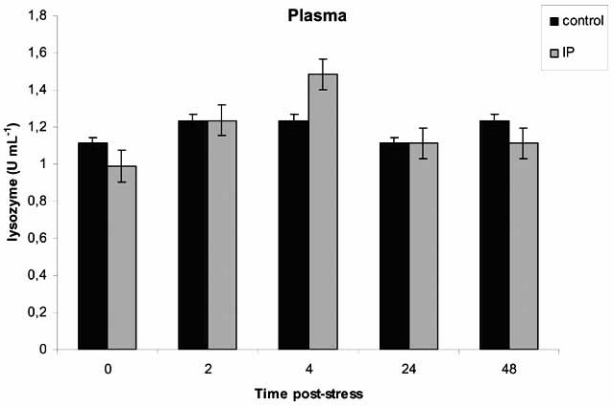

Fig. (5). Lysozyme content (in Units mL^{-1}) in the plasma of control and stressed (IP) juvenile sea bass. Values are means ± SEM (n = 8).

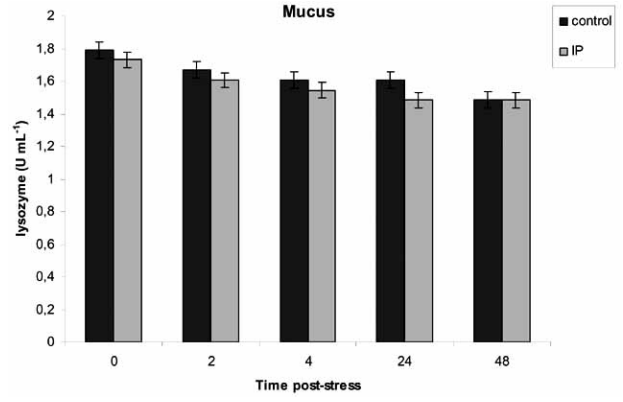

Fig. (6). Lysozyme content (in Units ml^{-1}) in the mucus of control and stressed (IP) juvenile sea bass. Values are means ± SEM (n = 8).

DISCUSSION

In aquaculture practices the understanding of fish stress response is essential to avoid stress-related problems, and to improve fish quality, optimizing productions.

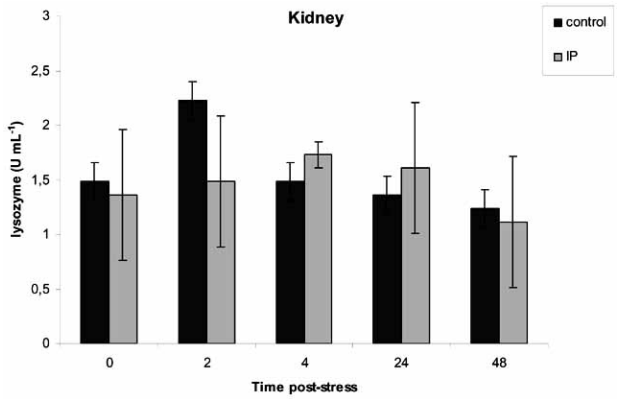

Fig. (7). Lysozyme content (in Units mL^{-1}) in the kidney of control and stressed (IP) juvenile sea bass. Values are means ± SEM (n = 8).

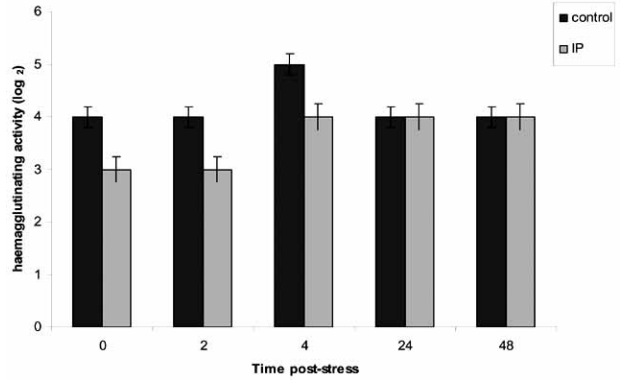

Fig. (8). Haemagglutinating activity (reported as log2 values of the final serum dilution showing visible agglutination) in control and stressed (IP) juvenile sea bass. Values are means ± SEM (n = 8).

Among the aquaculture practices that may cause stress intraperitoneal vaccination should be taken into consideration because of its potential adverse effects.

Much attention has been focused on the effects of vaccination upon growth, feed intake and feed conversion efficiency [32-39] and on tissue lesions and intra-abdominal adhesions [32, 34, 35, 40, 41]. To date, no study has been performed on physiological stress response.

To our knowledge, this experiment is the first performed in sea bass dealing with the study of physiological responses following intraperitoneal vaccination simulating an acute stress condition.

As in other vertebrates, fish experiencing stress show a number of physiological changes that are expressed through a number of particular indicators [2].

In the present study, serum cortisol was measured as primary stress response, serum glucose, haematocrit and haemoglobin as secondary stress response, and lysozyme and haemagglutinating activities served as measures of innate immune response.

The specific literature shows that the stress responses in fish varies both with the nature of the stress (acute or chronic) and with the species. Also, in the case of an acute stress, the

time course of stress response depends on the severity and duration of the stress itself [42].

Haematocrit and haemoglobin levels are frequently used as secondary stress haematic indicators [2, 43, 44]. However available data are often conflicting, depending on the type of stress imposed.

Tort *et al*. (1994) [45] and Altimiras *et al*. (1994) [46] reported a decrease of haematocrit value, in response to hypoosmotic shock, in sea bream, *Sparus aurata*. On the other hand, Benfey & Biron (2000) [47] showed that acute stress causes an elevation in haematocrit value, in contrast with above results. Moreover, Mazur & Iwama (1993) [48] reported no changes in haematocrit contents in chinook salmon subjected to handling stress.

Usually haemoglobin concentration seems to be less influenced by acute stress and no changes in this parameter were found as a consequence of acute handling in different fish species [47, 49, 50]. In the present study, no significant variations in these parameters was observed in juvenile sea bass subjected IP vaccination compared to control fish.

The present data support the studies where it is clear that these haematological indicators of secondary stress response are mainly displayed in chronic stress situations [24]. It has been shown that high stocking density, considered as an aquaculture-related chronic stressor [43] produce on juveniles of gilthead seabream, haemoconcentration, as a strategy for increasing oxygen carrying capacity of blood during periods of high energy demand [51-53].

Blood cortisol levels are widely used as an indicator of stress condition [2] because of the extreme sensitivity of the hypothalamo-pituitary-interrenal (HPI) axis.

In fish acute stress elicits increases in corticosteroid levels [18, 54, 55].

In general the cortisol response is transient and the time required for the circulating levels of this hormone return to normal depends on the intensity of the stressors [41].

In this study, IP injection provoked a characteristic acute stress response in sea bass, with cortisol peak reaching 620.45 ng mL^{-1}, 4 h after the stress.

Our results, also, show that a period of 48 h was not sufficient enough to fish to recover to pre-stress cortisol levels.

In other teleosts, such as *Sparus aurata* [8, 55, 56], *Salmo trutta* [6] and *Oreochromis niloticus* [57] the same time period is sufficient to observe a return to normality.

In addition, the present study gave the opportunity to observe the daily variation of cortisol levels in captive juveniles sea bass over a 24 h period (control group).

Daily variations of plasma cortisol in sea bass have been reported to be mainly associated with feeding times or with seasons [58, 59].

Planas *et al.* 1990 [59] compared daily changes of cortisol during four different months of the year and reported significant daily variations of this parameter in September when the water temperature reached the highest value (25.7 – 27.2°C). Our study was performed in June when water temperature was 24-25°C; in juvenile sea bass cortisol fluctuated daily from 100 to 200 ng mL^{-1} and these values as well as water temperature were similar to those reported by the Authors cited above.

Blood glucose levels have been also described to be affected by acute and chronic stress [2, 43, 60].

The response of sea bass to IP injection consisted of a hyperglycaemia 2 h after stress, while hypoglycaemia began after 4 h, and lasted for 48 h post-stress.

The rapid rise in glucose levels found in juvenile sea bass has been demonstrated in other teleosts exposed to acute stressors [6, 24, 31, 61].

The hyperglycaemia observed in these studies has been shown to be mediated by catecholamines [3, 62], which stimulate glucose release from liver, through glycogenolysis and gluconeogenesis [62-64].

Several factors (rearing history, nutritional status, environmental temperature and species) can affect fish stress response and glucose clearance rate [65].

The hypoglycemia observed in sea bass at 4 h post-stress is difficult to explain.

This might be ascribed to a particularly sensitive compensation process of blood glycemia which causes a rapid fall of glucose levels. Hypoglycemia, in turn, triggers the production of inhibitor hormones, such as glucagone, adrenaline and corticosteroids, which stimulate liver (and other organs) to release reserves to increase, again, glycaemia. This could explain the increase in cortisol observed 48 h after stress which corresponds to a rise in blood glucose.

Concerning the effects of IP vaccination on non-specific immune response parameters, it is generally accepted that in fish stress causes decreased immune function and increased susceptibility to disease, although the relationships between stressors, immune reactivity and disease incidence are still poorly understood [66].

In *D. labrax,* intraperitoneal vaccination caused increased lysozyme levels in plasma, whereas an opposite trend was observed in mucus and kidney. No significant differences between stressed and control fish were detected 4 hours after intraperitoneal vaccination.

In literature, the physiological response of fish to acute stress conditions in terms of non-specific immune response is still poorly investigated and only a few studies have focused on the effects induced on lysozyme activity by stressors such as handling or transport, frequent in fish culture [10, 66].

Lysozyme is an important enzyme involved in the non-specific immune response of many fish species [10]; it acts as an important factor in protecting fish against bacterial pathogens, due to its antibacterial properties against both Gram positive and negative bacteria.

Lysozyme content decreased in rainbow trout exposed to severe stress (such as that caused by transport), while it increased following less stressful conditions, indicating that the behaviour of this parameter may be different according to the strength of the stressor and the condition of each individual [66].

In rainbow trout, acute stressors such as handling, confinement and transport lasting 10 minutes were associated with significant increases in lysozyme activity in the serum, in agreement with what found in our study [67].

In rainbow trout subject to acute stress induced by bleeding an increase of lysozyme was noticed within 1 h [19], suggesting that acute stress may determine short-term enhancement of this defence molecule, in contrast to chronic stress, which is usually immunosuppressive.

Assuming that changes in lysozyme activity reflect the modulation of the defence system, the peak displayed by lysozyme, in kidney and plasma, 4 hours after intraperitoneal injection could suggest its initial activation, which is followed by its decrease to initial levels, similarly to what previously found in rainbow trout [66,19].

The increase in lysozyme levels is related to the cells that are responsible for its secretion; increased numbers of leucocytes were also observed in the head kidney of juvenile coho salmon (*Oncorhynchus kisutch*) [68] within 24 h of the stress.

In contrast, in Atlantic salmon IP vaccination [69] was found to elicit a stress response, consisting in the reduction of antibody producing cells and lymphopenia, whose duration exceeded that elicited by other acute stressors such as holding in a dip-net for 30 s [70] or crowding [6].

With respect to the haemagglutinating activity, agglutinins are recognised to be a key component of innate immune system in many fish species, which are active against homologous or heterologous red blood cells [8,10].

The trend shown in this study by the haemagglutinating activity values suggests that this parameter fluctuated with no apparent relationship with the induction of acute stress.

The increase recorded 4 hours after intraperitoneal injection is in contrast with previous research showing the progressive decrease in haemagglutination activity in gilthead seabream juveniles exposed to repeated acute stress consisting in chasing with a net for 5 minutes over 14 days [7, 71]. In the same species, chronic stress induced by crowding caused immunodepression, as suggested by decreases in haemagglutination titre observed after 9 days [72].

CONCLUSIONS

This study contributes to current knowledge on fish response to stress experienced as a consequence of aquaculture practices by adding some data on physiological effects similar to those produced by vaccination practice.

According to the time course of the parameters assayed in the present investigation it can be concluded that in *Dicentrarchus labrax* the acute response is commonly associated with the increase in cortisol and glucose levels, while it is not reflected by a clear response in terms of non-specific immune parameters, as suggested by the lack of statistical differences in lysozyme and haemagglutinating activities between stressed and control fish.

Results obtained show that juveniles of sea bass subjected to an intraperitoneal injection experience acute stress as demonstrated by neuroendocrine system activation.

The length of experiment did not allow us to observe sea bass recovery to normality suggesting that the physiological response of sea bass is significant enough to determine variations in overall welfare status. However further study is needed to determine how long the stress response lasts.

Our results confirm that the time needed for complete recovery is species-specific and is a function of duration and severity of the applied stress. Hence the need to know how each species react to routine practices in aquaculture.

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