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Seabream (*Sparus aurata*) long-term dominant-subordinate interplay affects phagocytosis by peritoneal cavity cells

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ABSTRACT

Fish are sensitive to stressful conditions that affect their innate immune systems and increase their susceptibility to diseases. We examined the social stress of paired gilthead seabream (*Sparus aurata*). Social hierarchies (dominant/subordinate) were characterised by behavioural changes, such as "aggressiveness" and "feeding order"; hierarchical positions were established within an hour of exposure to social stress and remained unchanged for approximately 1 year.

To characterise physiological stress, we measured blood plasma levels of cortisol, glucose, and lactate as well as osmolarity and observed that the levels of these stress markers were higher in subordinate individuals than in dominant ones. The discriminant analysis revealed a separation of the subordinate fish groups, and at 15 days, a significant separation among groups was observed.

Moreover, diminished phagocytic and respiratory burst activities revealed that social stress appeared to affect the cellular innate immune response of the subordinate specimens.

Finally, to examine the effect of cortisol on phagocytosis, peritoneal cavity cells were treated *in vitro*, and an inhibitory effect was observed.

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1. Introduction

In fish, social defeat refers to losing a confrontation among conspecific animals, is a powerful stressor that can lead to drastic changes in animal physiology and behaviour (Sloman and Armstrong, 2002; Martins et al., 2006; Fernandes-de-Castilho et al., 2008; Edeline et al., 2010). For socially dominant individuals, the "winner effect" underlies a higher probability of winning subsequent aggressive encounters (Rutte et al., 2006). In contrast, the lack of control of socially subordinate fish might result in increased cortisol levels and tertiary stress effects (Galhardo and Oliveira, 2009). Social interactions establish hierarchies in which the dominant fish occupy the most profitable positions (Sloman and Armstrong, 2002). Behavioural effects include appetite inhibition (Kramer et al., 1999; Meerlo et al., 1997; Øverli et al., 1998), reduced aggression (Blanchard et al., 1995; Höglund et al., 2001), decreased reproductive behaviour (D'Amato, 1988; Perret, 1992), and enhanced submissive and defensive behaviours toward conspecifics (Blanchard et al., 1993; Siegfried et al., 1984). Moreover, contact between conspecific fish does not promote habituation (Fernandes-de-Castilho et al., 2008). Changes in aggression levels and stress physiology are used as welfare indicators, and the elevation of these parameters has been associated with impaired welfare (FSBI, 2002).

The paired fish model is used to examine dominance hierarchies. In paired rainbow trout, fish position within a tank, locomotor activity, agonistic behaviour, feeding, and plasma cortisol levels have been reported as criteria for social dominance (Pottinger and Carrick, 2001); changes in plasma cortisol levels appeared to be a heritable trait. Cortisol exerts permissive, suppressive or stimulatory actions in various animals (Golub et al., 1979; Sapolsky et al., 2000), and it seems to correlate with the likelihood of fish establishing dominance (Sloman et al., 2001).

In fish, the primary response to stress involves the activation of the hypothalamic-pituitary-interrenal axis, which leads to increased levels of adrenocorticotropic hormone, cortisol, catecholamine and several plasmatic parameters and physiological activities, including the immune response (Fevolden et al., 2002; Vizzini et al., 2007; Vazzana et al., 2008, 2010). In aquaculture stress induced by confinement and by a high-density population increases blood plasma levels of cortisol, glucose, and osmolarity and affect innate immune responses.

Stressors diminish the respiratory burst and cytotoxic activity of leucocytes in the head kidney and peritoneal cavity of fish (Vazzana et al., 2002). Moreover, *in vivo* and *in vitro* experiments

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showed that increased cortisol levels affect phagocytosis via the cytosolic receptor DlGR1 in sea bass (Vizzini et al., 2007). This receptor was localised to the head kidney, spleen, gills, intestine, heart and liver tissues (Vazzana et al., 2008).

Peritoneal cavity cells (PCCs) play important roles in both non-specific and specific immune mechanisms (Ellis, 1982; Sakai, 1984; Suzuki, 1986; Olivier et al., 1992), and the peritoneal exudate has been used as a source of leucocytes for studies of innate immunity, including phagocytosis (Bodammer, 1986; Vazzana et al., 2003) and cytotoxicity (Cammarata et al., 2000).

In this paper, we examine the *Sparus aurata* social stress using paired fish. After the hierarchical positions (dominant or subordinate) were identified according to "aggressiveness" and "feeding order", the blood plasma levels of cortisol, glucose, lactate as well as osmolarity and leucocyte phagocytosis and chemiluminescence activities in peritoneal cavity cells were examined. The results showed that social stress affects the PCC response in subordinate individuals as revealed by a significant decrease in phagocytosis and respiratory burst activity.

Moreover, we showed that the *in vitro* treatment of PCCs with cortisol affects both activities.

2. Materials and methods

2.1. Animals

Seabream (*S. aurata*) specimens (n.116) weighing 150 ± 20 g were obtained from a commercial fish farm (Ittica Trappeto, PA). All individuals were reproductively immature males with no apparent differences in social history. The experiments were performed in full compliance with the national rules (D.Lgs 116/92 and subsequent amendments) and the international European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC).

After an acclimatising period of 1 week, the animals were subdivided into the following groups: 14 fish maintained at one per tank (100-L indoor glass aquarium) were used as controls, and 56 maintained at two per tank formed 28 experimental pairs. The 28 experimental pairs were further separated into four groups, constituting seven experimental pairs. Each group was examined at different times: 24 h, 15 days, 1 and 6 months after hierarchical establishment.

2.2. Tank conditions and behaviour observation

The tank water was examined daily and maintained at an average temperature of 18 ± 1 °C, 38 ± 1% salinity, >6 mg/L of oxygen and a nitrite concentration of <0.2 mg/L under a photoperiod of 12 h dark and 12 h light. The fish were fed a commercial pellet diet.

The paired fish were distinguished by unmodified morphological differences, such as opercular spots, mouth abrasions, facial structure and possible small external alterations of the tail, dorsal or pectoral fins.

The behavioural changes were recorded by using two cameras and a digital multifunctional system for the data acquisition (DR41).

To distinguish the dominant and subordinate individuals, the pairs were recorded for 12 h. After the social positions were established, every pair was observed for 1 h per day to detect changes in the social status.

The individual behaviour was examined by the continuous survey of the different behaviour categories (Martin and Bateson, 1988), and individuals from each pair were distinguished according to McCarthy et al. (1999) as dominant, called "α", or subordinate, "β". High social status has been correlated with increased aggressiveness and preferential access to the food (Johnsson, 1997; McCarthy et al., 1999). To define this social distinction, the number of aggressive acts (A+) were observed and defined as a bite or a rapid approach without biting that resulted in the displacement of the subordinate (Øverli et al., 2002), and the feeding order (FO) of each pair was determined.

2.3. Cell suspensions

The fish were anaesthetised with 0.05% w/v MS222 (3-aminobenzoic acid ethyl ester) (Sigma) in seawater. Blood was collected from the Cuvierian sinus heart into a sterile plastic syringe containing 0.2 ml of heparin and centrifuged at 800*g* for 10 min at 4 °C.

The peritoneal cavity cells (PCCs) were obtained as follows: subsequent to disinfection of the body ventral surface with 70% ethyl alcohol, the peritoneal cavity was injected with 15 ml of isotonic (370 mOsm kg^{-1}) medium (Leibovitz L15 medium containing 2% foetal calf serum, 100 units penicillin ml^{-1}, 100 units streptomycin ml^{-1} and 10 units heparin ml^{-1}). The culture medium components were purchased from Sigma.

After massaging the ventral surface for 10 min, the medium containing the PCCs was collected, and the PCCs were isolated by centrifugation at 400*g* for 10 min at 4 °C.

The dead cells were determined by light microscopy after addition of 0.01% trypan blue to the medium.

2.4. Haematological parameters

The levels of total cortisol were measured in the plasma samples using a commercially available kit (Sentinel diagnostics SRL) according to the manufacturer's instructions and confirmed by radioimmunoassay (RIA) (Espelid et al., 1996).

The osmolarity of the plasma samples was measured using a freezing-point depression osmometer (Roebling). The glucose plasma levels were determined using the Accutrend GC Kit (Roche) according to the manufacturer's instructions. The lactate plasma levels were determined using a commercially available kit (Sentinel Diagnostics SRL) according to the manufacturer's instructions.

2.5. Leucocyte smears

Samples (50 µl) of the leucocyte suspension (5×10^6 cells ml^{-1}) were layered on a slide, allowed to settle for 30 min and fixed with undiluted methanol for 7 min at 4–8 °C. The fixed smears were treated with May-Grünwald for 3 min, and after washing, the smears were further treated with May-Grünwald-Giemsa stain (MG-G) for 10 min. After ethanol dehydration and xylene treatment, the slides were mounted in EUKITT (Sigma). Leucocyte types were classified according to Meseguer et al. (1994).

Differential counts were performed on slides from 15 fish by examining more than 400 cells.

2.6. Chemiluminescence (CL) assay

The activation of phagocytosis was measured by the enhanced production of reactive oxygen intermediates (ROI) using a luminol-amplified CL assay. Luminol was prepared as a potassium salt by adding 0.014 g luminol, 0.78 g potassium hydroxide and 0.618 g boric acid to 10 ml of distiled water. The luminol solution was stored at 4 °C in the dark and diluted 1:100 in Hank's Buffered Salt Solution (HBSS) to obtain a working solution. Zymosan, a β-glucan of the yeast wall, was prepared by boiling for 30 min in phosphate buffered saline (PBS) followed by centrifugation for 5 min at 400*g*. The pellet was resuspended in 50 ml of PBS and stored at 4 °C until further use; the maximum storage time was

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Table 1
Mean percentage (±SD) of aggressive acts (A+) and preferential access at the food (FO).

	Aggressive acts Mean% ± SD	Preferential food accession Mean% ± SD
Dominant (α)	85.7 ± 5%	100 ± 0%
Subordinate (β)	14.3 ± 5%	0 ± 0%

2 weeks. In accordance with Scott and Klesius (1981), the final concentration of zymosan was 1 mg ml^{-1}.

The cell suspensions were standardised in HBSS containing 2.5×10^5 cells ml^{-1}. Aliquots of 0.5 ml of leucocyte suspension were mixed in propylene vials with 0.5 ml luminol (working solution). The reaction was initiated by the addition of 0.5 ml of zymosan stimulus (Scott and Klesius, 1981). In the control samples,

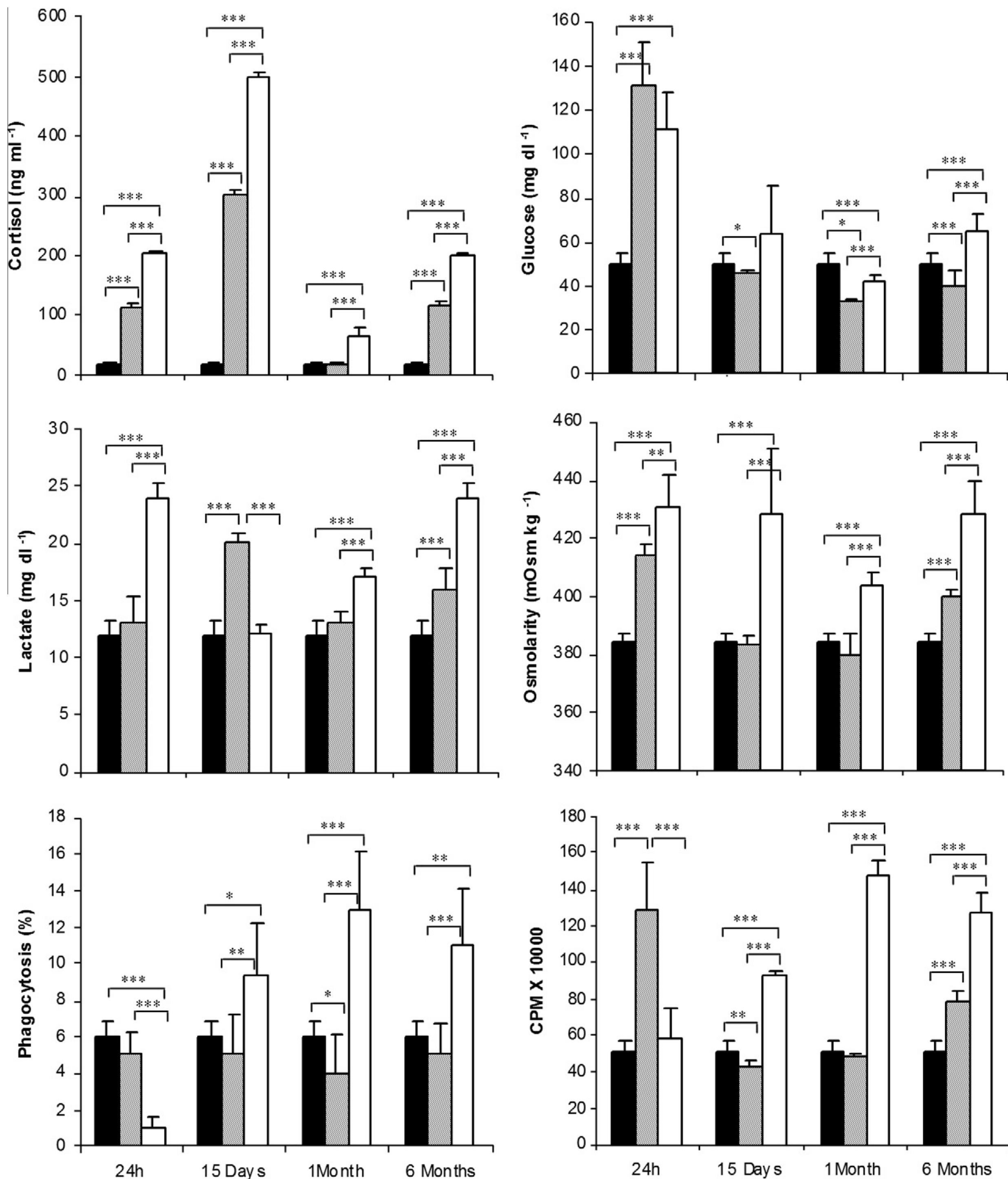

Fig. 1. Mean plasma and cellular activity values (±SD) time courses, prior to, and post hierarchical position establishment of: (a) Cortisol concentration, (b) Glucose concentration, (c) Lactate concentration, (d) Osmolarity level (e) Chemiluminescence; (f) Phagocytosis. ANOVA *P* comparison among treatments are showed as *($P < 0.05$); **($P < 0.01$), ***($P < 0.001$). Control fish: ■; Dominant Fish: ▨; Subordinate Fish: □ .

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the cells, luminol or zymosan in the reaction mixture were replaced by medium. The samples were run in triplicate. A liquid scintillation counter (LS 1800, Beckman) was used to measure the CL.

2.7. Phagocytosis fluorescence quenching in vitro assay

The method established in Cammarata and Arizza (1994), using *Saccharomyces cerevisiae* (Sigma) as a target, was performed with slight modifications. Briefly, yeast were prepared in distiled water as a 0.25% (w/v) solution (approximately 1×10^7 yeast ml^{-1}), autoclaved for 15 min, washed 2 times at 2000*g* at 4 °C for 5 min and incubated for 1 h at 20 °C with eosin Y (4-Bromo-fluorescein) to a final concentration of 0.05%. The yeast were washed four times in distiled water and resuspended to a final concentration of 0.0125% w/v in phosphate buffered saline (PBS: 103.6 mM NaCl, 1.46 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 2.6 mM KCl, 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$, pH 7.4) and stored at −20 °C for a maximum of 2 weeks.

The yeast suspension was added (v/v) to 100 μl of leucocyte suspension (2.5×10^6) and placed in a 1 ml plastic tube. The mixture was incubated for 30 min at 20 °C with gentle stirring. To indicate the activation of phagocytosis, 50 μl of a quenching solution (QS) (2 mg ml^{-1} trypan blue and 2 mg ml^{-1} crystal violet in 0.02 M citrate buffer, pH 4.4 containing 33 mg ml^{-1} NaCl) (Hed, 1986) was added to the reaction mixture. The slides were examined under a microscope equipped with a Normarski interferential contrast device and fluorescence apparatus (450–490 nm filter) (Diaplan, Leika, Wetzlar, D). A total of 200 35 cells on each slide were counted at an 800× magnification. The results were expressed as a percentage of cells containing yeasts.

To study the possible effect of cortisol on leucocyte activities, PCC leucocytes were incubated with or without (control) different concentrations of cortisol (Hydrocortisone, Sigma). A 1-ml aliquot of leucocytes (adjusted to 2.5×10^5 cells/ml) was dispensed into each well of a flat-bottomed 48-well microtitre plate (Nunc) and incubated without cortisol (control) and with 10^{-7}, 10^{-6} and 10^{-5} M cortisol for 60 minutes at 20 °C in 85% relative humidity and a 5% CO_2 atmosphere.

2.8. Statistical analyses

To predict group membership, the discriminant function analysis was used to determine the separation of variables among two or more naturally occurring groups, and to determine whether groups differ with regard to the mean of a variable. To evaluate which of the many variables contributed to the discrimination between groups, a matrix of total variances and covariances was obtained. To determine whether significant differences (with regard to all variables) between the groups existed, the matrices were compared via the multivariate *F* test. This calculation is identical to the multivariate analysis of variance (MANOVA).

The multivariate test was first performed, and if statistically significant, the variables with significantly different means were furthered analysed using "Statistica" (Statsoft) software. In addition, to examine the intercorrelation between two sets of variables, canonical correlation analysis have been performed for a multiple group of discriminant analysis. Repeated measures and ANOVA analyses followed by *post hoc* Student's *t*-test were used to assess differences between dominant and subordinate responders. A probability level of $p < 0.05$ was considered significant.

Since the values from the control individuals, recorded at each time point, did not show any significant difference, data from the experimental group were compared to the average value resulting from 14 control specimens.

3. Results

3.1. Dominance and dominance determination

As shown in Table 1 the percent of aggressive acts (A+) and preferential access to the food (FO) distinguished ($p < 0.001$) the fish as either dominant (A+ = 85.7 ± 5%; FO = 100 ± 0%) or subordinate responders (A+ = 14.3 ± 5%; FO = 0 ± 0%), "α" and "β", respectively, in each pair. This distinction remained unchanged for the duration of the experiment in all pairs of fish. No fish died or showed signs of disease during the experiment.

3.2. Cortisol, glucose, lactate and osmolarity evaluation

The plasma cortisol levels recorded at different time points in the control, α and β fish are listed in Fig. 1. At 24 h, a significantly increased plasma cortisol level was observed among the subordinate fish in both social categories. In particular, 24 h after the establishment of hierarchical positions, a significant difference in cortisol concentrations between control (18 ± 3 ng ml^{-1}), paired (α: 114 ± 5 ng ml^{-1}; ($F(1,11) = 1530.9$ $p < 0.001$; β: 204 ± 4 ng ml^{-1}; ($F(1,11) = 10010$, $p < 0.001$) were found. After 15 days, the cortisol concentration reached a maximum value in both the social categories (α: 302 ± 8 ng ml^{-1}; β: 500 ± 6 ng ml^{-1}). However, after 1 month, the cortisol levels in the dominant fish returned to the basal levels (α: 18 ± 3 ng ml^{-1}), while significantly higher cortisol levels were maintained in the subordinate specimens (β: 66 ± 12 ng ml^{-1}; ($F(1,11) = 86.6$ $p < 0.001$). After approximately 6 months, although the cortisol values were beginning to decline, the observed levels were higher than those observed at 1 month (α: 118 ± 4 ng ml^{-1}; ;($F(1,12)$=3485.1, $p < 0.001$); β: 199 ± 5 ng ml^{-1} ($F1,12) = 699.8$, $p < 0.001$).

At 24 h after the hierarchical position was established, both paired fish displayed a significant 2-fold increase in their plasma glucose level (Fig. 1). Although, at 15 days, these values returned to the control level, the glucose value of the β fish was consistently higher than that of the dominant fish. For the duration of the experiment, the lactate concentrations of the dominant fish remained almost at the control level (13–16 mg dl^{-1}), whereas 2-fold increases were observed in the β fish showed (24 ± 1 mg dl^{-1}; $F(1,11) = 255.9$, $p < 0.001$), (Fig. 1).

The plasma osmolarity values at 24 h in both fish categories were higher (α: 414 ± 4 mOsm kg^{-1}; ($F(1,11) = 245.7$ $p < 0.001$; β: 431 ± 11 mOsm kg^{-1} ($F(1,11) = 108.64$ $p < 0.001$); than that of the control (384 ± 3 mOsm kg^{-1}). However, from day 15 to 6 months, the osmolarity level in the dominant fish returned to basal levels, while in the subordinate fish it remained significantly higher.

3.3. Discriminant analyses of haematological parameters and immune activities

As shown in Fig. 2a, the plot of the canonical axes, corresponding to the data with respect to the hierarchical position, showed that all of the fish from the β groups formed a cluster independent from that of the α and the control fish groups (squares represent the canonical correlation 0.839 $p < 0.001$).

Fig. 2b shows a significant separation among the social groups at different times after pairing (squares represent the canonical correlation 0.999; $p < 0.001$) in which the groups were clearly separated. The dominant fish were affected by the new social status only in a first phase, whereas the subordinates suffered more significant social and physiological changes as demonstrated by the stress parameters (Fig.1). In any case, the greatest effects occurred within the first 15 days after the pairs were formed.

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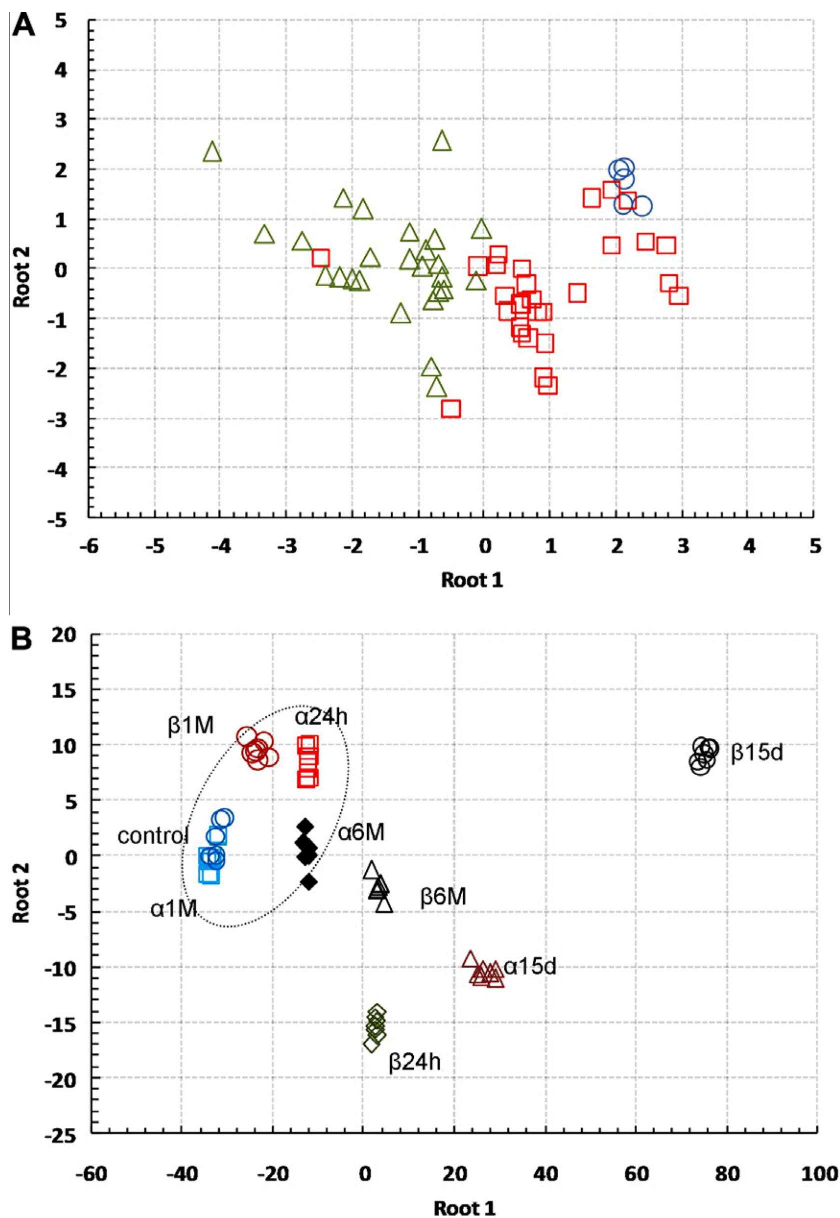

Fig. 2. Scatter plot of the canonical discriminant analysis of haematological parameters and immunological activities. (a) Effect of the *Sparus aurata* social status. ◯ Control; □ dominant; ◇ subordinate. (b) Effect of the *Sparus aurata* social status at different time after pairing. Groups: ◯ control; □ α24 h; ◇ β24 h; △ α15d; ◆ β15d; □ α1 M; ◯ β1 M ◆ α6 M; △ β6 M. (α) Dominant; (β) Subordinate; (h) hours; (d) days; (M) months. Cortisol and phagocytosis values were discriminant along the *X* axis (Root 1) between the subordinate and dominant fish groups during the entire pairing time. In the *Y*-axis (Root 2), osmolarity, phagocytosis and chemiluminescence contribute to the discrimination.

The canonical axes 1 and 2 of the discriminant analysis explain approximately 95% of the variability between the groups and the significant influence of the pairing time. Along root 1 (*X* axis), the cortisol and phagocytosis values were discriminant for the effect of the social stress for the entire duration of the experiment (6 months). Along the *Y*-axis, osmolarity, phagocytosis and chemiluminescence also contribute to the discrimination between the subordinate and dominant fish groups during the entire pairing time.

3.4. Peritoneal cavity cells (PCCs) identification

In PCCs preparations, the following cell types were observed: (1) Granulocytes, which were the most abundant cell type identified as eosinophils (GI) and neutrophils (G II) by MG-G stain (GI: 33.53% ± 1.3 and G II: 33.65% ± 2.5); (2) Lymphocytes (25.0% ± 1.2), which presented a large round blue nucleus after staining; and (3) Macrophages (7.53% ± 1) as observed by MG-G stain (nearly azure, large vacuoles, irregular cell surface with finger-like projections).

3.5. In Vitro phagocytosis and chemiluminescence assay

Fig. 3 shows typical phagocytes with internalised yeast as observed with a Normarski contrast interference microscope (Fig. 3a and b) or after MG-G staining (Fig 3a, spot). In Fig. 1, the percent values of the PCC phagocytes are shown. The values in control and α fish were unchanged throughout the entire experimentation period (5–6% ± 1). However, at 24 h after pairing, the PCCs from the subordinate fish showed significantly decreased phagocytic activity

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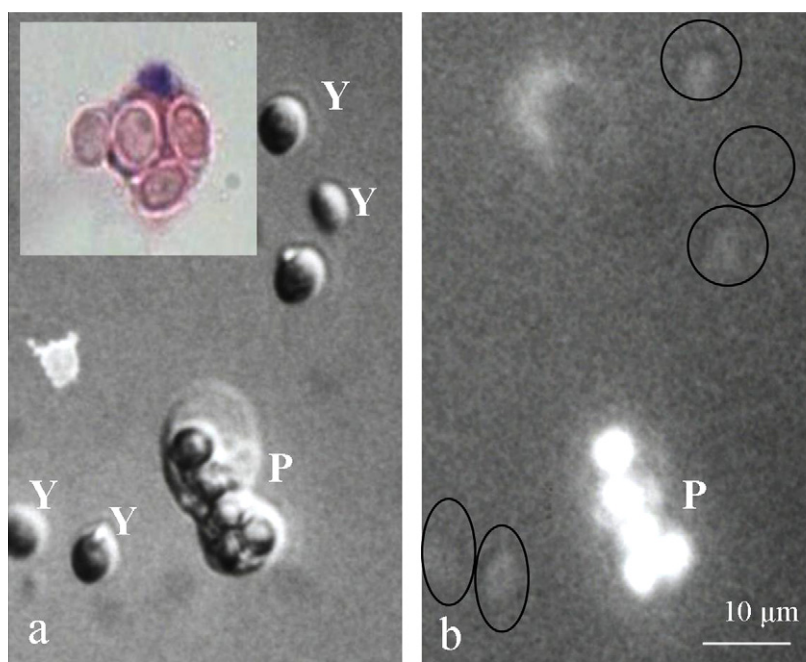

Fig. 3. *Sparus aurata* leukocyte phagocytosis observed in darkfield phasecontrast and in fluorescence: (a) Nomarsky contrast interference observation, Y: eosinY pre-treated yeasts; P: phagocyte containing eosinY pre-treated yeasts; (b) in the same panel, UV-light observation, P: phagocyte containing eosinY pre-treated yeasts; rings: quenched yeasts; spot) light microscopy observation of a typical phagocyting cell stained with May-Grünwald-Giemsa.

(1% ± 1; ($F(1,11) = 148.08$ $p < 0.001$), and at 15 days, this activity was almost doubled (9% ± 3). The activity was further increased in the subordinate individuals, reaching 13% ± 3 ($F(1,11) = 27.21$ $p < 0.001$); at 1 month and 11% ± 3 ($F(1,11) = 13,88$ $p < 0.01$); at 6 months.

Although the CL was proportional to the recorded phagocytic activity levels (Fig. 1), at 24 h a significant ($p < 0.001$) increase in the CL activity in the PCCs of dominant fish ($129 \pm 26 \times 10^4$ cpm) was observed as compared with the control ($51 \pm 5 \times 10^4$ cpm ($F(1,11) = 51.79$) and subordinate ($58 \pm 17 \times 10^4$ cpm ($F(1,12) = 37.37$) fish. Subsequently, the values in the α groups decreased to control levels, whereas the β fish showed a significant increase in CL activity from 15 days to 1 ($F(1,12) = 300,32$ $p < 0.01$); and 6 months ($F(1,12) = 64,74$ $p < 0.01$).

3.6. Cortisol affects the CL response in the head kidney and the peritoneal cavity leucocytes stimulated with zymosan

To examine the effect of cortisol on the CL response in PCCs, *in vitro* assays were performed by incubating 2.5×10^5 cells ml^{-1} (1 h) with two hormone different concentrations. A significant dose-related decrease in CL ($p < 0.001$) was observed. The zymosan treated cells in the absence of the hormone were used as controls.

As shown in Fig. 4, the respiratory burst and phagocytic activity were significantly modulated by increasing cortisol concentrations. The CL response was significantly decreased in the presence of 10^{-6} and 10^{-5} M cortisol ($p < 0.001$). A similar effect on the phagocytic activity was observed ($p < 0.05$ and 0.01, respectively).

4. Discussion

Social defeat could be a powerful stressor that can lead to dramatic alterations in behaviour and fish physiology. To examine changes in social stress and physiological parameters, experiments using paired fish disclosed hierarchical positions (dominant (α)/(β) subordinate) as observed by changes in aggressiveness as defined by Øverli et al. (2002) and in the feeding order. In this paper, dominant and a subordinate fish were identified among paired immature gilthead (*S. aurata*) males (28 pairs). This hierarchy was established within an hour and remained unchanged for approximately 1 year. Almost immediately after pairing (at 24 h), a significant increase in blood plasma levels of cortisol, osmolarity, glucose and lactate were observed in both dominant and subordinate fish. However, the cortisol, osmolarity and lactate values recorded in the subordinate fish were significantly higher than those observed in the α fish. The glucose levels were increased in both (α) and (β) fish, but higher values in the dominant (α) fish were observed. The secondary responses may be caused by the endocrine response. Cortisol plays a role in stimulating some processes (e.g., gluconeogenesis) and inhibiting others (e.g., digestion). According to Peters et al. (1980) the elevated plasma glucose, originated from hepatic glycogen, could indicate the mobilisation of energy to maintain the dominant hierarchical position. In this respect, measurable changes in blood lactate as a product of the anaerobic metabolism, suggest that energy could be detracted from muscle tissues and reproductive system (Dinkel et al., 2002).

Since the increased glucocorticoid levels exhibited during stress might also serve to restrain defence mechanisms (Øverli et al., 2004) we examined the relationship between the social rank and the effects on the innate immunity. In particular the activity in the peritoneal cavity cells was estimated using a phagocytosis activity assay employing yeast (PA) and a cell chemiluminescent (CL) reaction subsequent to stimulus with zymosan. The CL response is due to the respiratory burst through ROS production that occurs in phagocytes after responding to a superficial stimulus, which facilitates an evaluation of phagocytic responses. In a previous paper, a decrease in both activities was related to confinement stress in sea bass (Vazzana et al., 2002).

After a 24 h pairing period, the PCC activity, with respect to PA and CL, was significantly higher in the dominant and control fish as compared with the subordinate ones. However, after 15 days, these responses returned to control levels in dominant fish, and these levels were maintained for 6 months. In contrast, in β animals, the PCC activity was increased by 3-fold within 1 month and 2-fold

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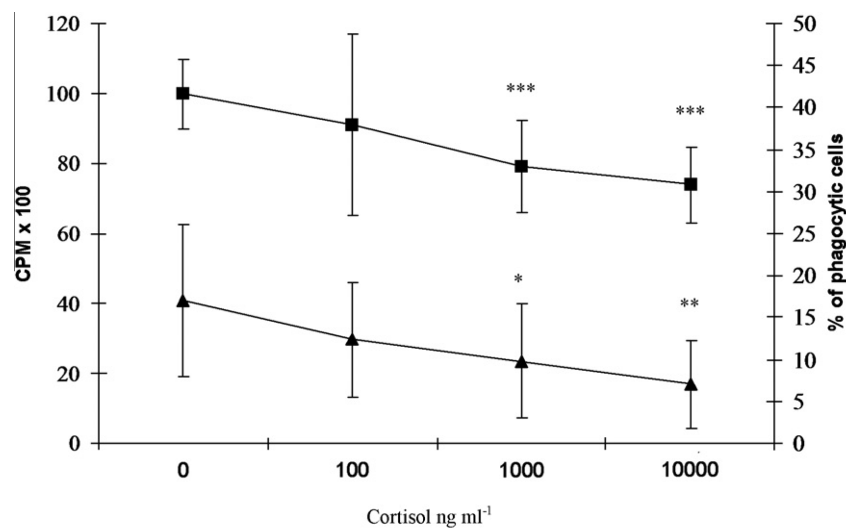

Fig. 4. Phagocytosis (■), and CL responses (▲) of leucocytes from the seabream peritoneal cavity after *in vitro* treatment with cortisol. The percentage of phagocytic cells and the respiratory burst activity of the PC leucocytes after 1 h of incubation with different concentrations of cortisol is shown. (Control: no cortisol; Cortisol 100: 10^{-7} M; Cortisol 1.000: 10^{-6} M; Cortisol 10.000: 10^{-5} M). Data are represented as the mean ± SD. *($P < 0.05$); **($P < 0.01$), ***($P < 0.001$).

after 6 months. Subordinate individuals could potentially upregulate PA and CL through an increased mobilisation of energy substrates, which lowers liver glycogen and reallocates energy away from immunity, growth and reproduction (Wendelaar-Bonga, 1997).

To establish the relationship between haematological and immune reactivity parameters (glucose, cortisol, osmolarity, lactate, PA and CL), a statistical evaluation was performed using a discriminant analysis of the data from all dominant, subordinate and control fish. During 6 months, as shown in the canonical root of the *X*-axis, the cortisol and osmolarity values were discriminant, and along the *Y*-axis, changes in glucose levels, and phagocytic and chemiluminescence activity distinguished fish affected by social stress. The influence of the social status was manifest at 15 days in the dominant fish, and within 1 month, the status returned control levels. The values that represent the dominant fish significantly change in the defined space of canonical roots as compared with the control fish. However, although the greatest effect was observed at 15 days, a clear influence was evident in the subordinate fish at 24 h that remained for 6 months. The subordinate fish group values were significantly discriminated in the canonical roots as compared with the control and α fish; these values were influenced differently in the roots at a different time. Fernandes-de-Castilho et al. (2008) showed that contact between conspecific fish constituted a chronic form of social stress; the increased plasma cortisol in socially subordinate *Oncorhynchus mykiss* and *Salmo trutta* has also been reported (Sloman et al., 2001).

To ascertain the cortisol effect on gilthead seabream PCCs, cells treated with three cortisol concentrations *in vitro*. We showed a dose-dependent significant decrease in phagocytic activity, the phagocytic index and CL activity in PCCs. Although, it is questionable whether an adequate comparison can be made between *in vivo* and *in vitro* responses, the *in vitro* assay indicates a close relationship between stress perceptions, the cortisol-mediated responses and phagocytosis. The results of cortisol treatment on PCCs *in vitro* produced a short-term response (1 h), and these responses were in inconsistent with the increased phagocytotic activity observed in these fish when maintained in chronic (6 months) stress conditions. Sorrells and Sapolsky (2007) have extensively discussed a number of molecular mechanisms to explain such an "inverse-U" GC effect in different types of stress responses, where increased cortisol promptly inhibited the immune system (anti-inflammatory effect). In addition, when a chronic exposure to cortisol occurred, a pro-inflammatory effect could be observed.

The possibility exists that the effect might be related to glucocorticoid receptors. Vizzini et al. (2007) showed that the increased cortisol level (1 h) *in vitro* decreased sea bass leucocyte CL via the binding to the glucocorticoid receptor DlGR1 (Vizzini et al., 2007). In mice, the expression of the glucocorticoid receptor mRNA was down-regulated in the brain and spleen due to the social hierarchy distruption (SDR) (Avitsur et al., 2001; Quan et al., 2001). When subordinate and dominant mice were examined, differences in the individual responses to SDR suggested that the subordinate social status played a key role in mediating the splenic immunological response (Avitsur et al., 2007).

In the present paper, the testosterone levels that can regulate cellular immune functions (Gesquiere et al., 2011) have not been evaluated. However, sea bream have an r-selection reproductive strategy as the ability to reproduce quickly and is a proterandrous hermaphrodite species that at this stages (here examined) are immature male. It is noteworthy that we examined a small fish group from a large group kept in a farm, and it is known that this species in the upbringing conditions does not show significant variation in the cortisol concentration (Arends et al., 1999). Apparently, the ranking experiments affected physiology and the innate immune responses here tested.

A striking result from this study was observed: the individuals that had rapidly resumed feed intake after pair formation nearly always won the subsequent fight for social dominance. In this context, it would be of interest to examine whether feeding behaviour in a breeding environment is a consistent individual trait that is predictive of other behavioural and physiological features.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2012.01.008.

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