

CHRONIC SOCIAL STRESS IN RAINBOW TROUT: DOES IT PROMOTE
PHYSIOLOGICAL HABITUATION?

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Abstract

The effect of chronic social stress on growth, energetic substrates and hormones was tested in rainbow trout, *Oncorhynchus mykiss*. After a 14-day isolation period, the fish were paired for 8 days. In order to expose fish to chronic intermittent social contact during pairing, they were maintained in direct contact with each other during the first day. After that, a black plastic screen partition was introduced in each tank, preventing direct contact between animals. Every day the partition was removed for 30 minutes, allowing physical interaction between fish. At the end of pairing period, they were isolated again for 13 days. Fish were weighed and blood was sampled frequently during the experiment. Plasma levels of cortisol, growth hormone, glucose, total protein and free amino acids were quantified. Both dominants and subordinates had specific growth rate decreased during the pairing period and but only subordinates increased when the stressor was abolished (dominants: 0.32 ± 0.21 and 0.24 ± 0.41 , subordinates: -0.77 ± 0.29 and 0.37 ± 0.31 , respectively). Dominants showed a higher cortisol level one week after pairing condition had been abolished than subordinates (dominants: 56.76 ± 13.26 , subordinates: 31.89 ± 13.36). We conclude that chronic condition of intermittent social stress represents a stressful condition for animals of both hierarchical ranks and a treatment of one daily short direct contact between conspecifics does not promote habituation in fish, as mentioned for other stressors.

social stress, energetic of stress, hierarchical dominance, rainbow trout, *Oncorhynchus mykiss*.

Introduction

The stress resulting from farming practices, such as temperature, handling, sorting and grading, has received considerable attention and been the subject of numerous reviews on fish (Adams, 1990; Barton and Iwama, 1991; Pickering, 1992; Ruzzante, 1994; Wandelaar Bonga, 1997). Recently, however, some authors have considered that these stressors have little heuristic value because they bear little or no relation to the environmental challenges an animal may face in its natural environment. From a biological standpoint, it seems that the social environment of a species can be a considerable source of stress (Blanchard et al., 1999; DeVries et al., 2003; Koolhaas et al., 1997). In many species, animals are organised in territories and a hierarchical rank of dominance is established. In such cases, social stress can be considered the stress resulting from the physical contact between animals (density and agonistic interaction) plus psychological components, like hierarchical instability and submission (Zayan, 1991).

Social stress can affect several aspects of vertebrate physiology. Most studies dealing with this subject were conducted on primates and small laboratory mammals and in acute conditions, e.g., when animals are kept above stressor stimuli for a short period of time and the behavioural and physiological responses are monitored during and shortly after the stressor had been removed (Chrousos et al, 1995; Csermely, 1998). In fish, some studies have been carried out using the same procedure and the results obtained were similar to those found for other vertebrates, with activation of the pituitary-

adrenal (PA) and sympathomeduloadrenal (SMA) axes (Flik et al., 2006; Wendelaar Bonga, 1997).

The social stress attributable to the interaction between conspecifics in their natural environment, or even on farms, can be considered much more a chronic than an acute stressor, according to the duration of exposure. If we consider that most animal species use some behavioural strategies, like flight or hiding, to reduce their direct contact with other individuals, the stressor can also be considered an intermittent one.

Animals exposed to chemical or physical stressors for long periods of time are described as displaying physiological habituation, even in the presence of the stimulus (Barton, 1997; Colombo et al, 1990). Volpato and Fernandes (1994) did not hypothesise that this happens in the same manner when animals are subjected to social stressors. Social interactions between conspecifics are, at least for some fish species, dynamic processes, where sub-dominants are frequently trying to become dominants and these latter ones are trying to maintain their dominant status, using direct attack or signs to the others. Thus, the aim of this study was to determine whether rainbow trout, *Oncorhynchus mykiss*, show habituation to chronic intermittent social stress in the context of fish hierarchical rank. To that end, a social intermittent condition of pairing was imposed chronically on juvenile rainbow trout, and some known stress indicators (i.e., growth rate, cortisol, growth hormone, glucose, total protein, free amino acids) were measured before, during and after the social contact was abolished.

Methods

The experimental design consisted of monitoring selected indices of stress in rainbow trout before, during and after exposure to chronic intermittent social stress of dominance.

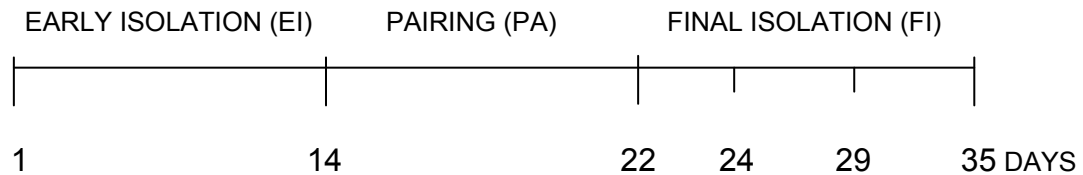
Sixteen 1-year-old immature rainbow trout, (Annandale strain, mean weight = 215 ± 33.5 g), were removed from the population of fish maintained in 1500-liter capacity outdoor circular fiberglass tanks, supplied with a constant flow of water (from Lake Windermere) at $30 \text{ litre min}^{-1}$ at ambient temperature. Fish were distributed individually among 16 75-liter indoor glass aquaria, also supplied with a constant flow of lake water (1.5 litre per min) at the same temperature as that in the holding tanks (Center of Ecology and Hydrology, Windermere Laboratory, UK). Water temperature during the experimental period was between 10 and 16° C. Fish were fed once daily with commercial pellets (BP Mainstream, BP Nutrition UK Ltd) at a rate of 3% of body weight. The photoperiod was maintained constant with light-dark cycle of 14h:10h. Fish were considered acclimated when all of them were observed feeding normally (more than 50% of the offered pellets), which occurred after 15 days under lab conditions.

After the acclimation period, all the isolated fish were removed from the troughs, rapidly anaesthetised (2-phenoxyethanol, 1:2000) and a blood sample was collected from the Curverian sinus (~ 0.5 ml) using 1.0-ml heparinized syringe. The blood was kept on ice until the completion of sampling when it was centrifuged (3000g at 4°C for 10 min). Separated plasma was transferred to a capped polypropylene tube and stored frozen (-20°C) until required for assay.

The fish were weighed before returning to their tanks. This moment was considered the starting point of the experiment.

Animals were maintained in isolation for a 14 days period (early isolation - EI) in order to get standard values of the measured parameters. Immediately after this procedure, ten pairs were formed, with fish having a minimum body weight difference of 10% between individuals within each pair. To allow for individual identification of the specimens used, each experimental fish was marked with aqueous alcian blue dye on its right or left ventral surface delivered by a Panjet needleless injector (Wright Dental Co.). Each pair was introduced into another 75-liter tank to avoid the establishment of a resident-intruder paradigm. In order to expose the fish to intermittent chronic social contact, fish were maintained in direct contact with each other for the first 24 hours of pairing. At the end of this time we could identify the dominant and subordinate fish. Dominant fish were considered those that presented bright colour, moved throughout the aquarium and displayed agonistic attacks against the other most of the time. Subordinate fish displayed dark colour, low locomotion activity and flight from the conspecific attacks. Thus a black plastic screen partition was introduced in each tank, preventing direct contact between the members of each pair. Every day during the afternoon, the partition was taken away for 30 minutes to allow physical interaction between animals (to reproduce intermittent social contact). Fish were fed daily in the morning before the partition was removed to avoid potential effects of stress from agonistic interactions and from handling the partition at feeding. Fish were maintained in this pairing condition (PA) for 8 days, at the end of which they were sampled using the same procedure described above, and returned to the isolation condition (final

isolation - FI), where they were maintained for another 13 days. During this 3rd. period animals are sampled more frequently in order to monitor the recovery responses after the stressor was abolished, as indicated.



Both plasma cortisol and growth hormone (GH) were determined by established, well-validated radioimmunoassays (Pickering and Christie, 1981; Rand-Weaver et al., 1992). Glucose levels were measured using a commercially available kit (SIGMA Diagnostics- quantitative, enzymatic [Glucose Oxidase] determination, procedure n^o. 510). Total protein concentration and plasmatic amino acids were quantified using a colorimetric method (Onhnishi and Barr, 1978; Matthews et al., 1964).

Changes in body weight are expressed as Specific Growth Rate ($SGR = [\ln W_f - W_i / \text{days}] \times 100$), where W_f and W_i represent the final and initial body weight in a period of days, respectively. Two-way repeated measures ANOVA followed by Tukey's post hoc multiple comparisons test was used to compare the effects of social status and experimental periods (early isolation (EI), pairing (PA) and final isolation (FI)) on growth parameters (SGR and ΔSGR) and cortisol, glucose, protein and amino acids secretion rates. From a plot of residuals against fitted values, appropriate transformations were selected to improve the homogeneity of variance (cortisol: $\text{sgrt}(x)$; glucose: $1.0/x$; protein: $\ln(x)$; amino acids: $1.0/x$). As it was not possible to find an appropriate transformation for GH values, Friedman's two-way analysis of variance followed by the LSD test was used to compare, individually, subordinate and

dominant animals on different moments. To compare mean GH levels between dominants and subordinates in each moment, Wilcoxon matched pairs test was used. For Specific growth rate, hormones and metabolic substrates secretions the Pearson Correlation Test was established. Statistical significance was set at 5% in all analyses.

Results

Changes in body weight from dominant and subordinate fish are shown in Fig. 1. Statistical analysis of specific growth rate (SGR - Fig. 1A) using a two-way repeated measures ANOVA followed by Tukey's post hoc multiple comparisons test revealed significant effects of experimental periods on SGR ($F = 10.25$, $P < 0.001$), and of social status on SGR ($F = 5.72$, $P < 0.05$). However, the interaction between moment and social status was not statistically significant ($F = 2.96$, $P > 0.05$), indicating that the pattern of growth specific rate remained different between dominant and subordinate fish throughout the experiment. Subordinate animals showed a negative specific growth rate (SGR) mean value at the end of 8 days of pairing, which was significantly different from the other moments (Fig. 1A: EI/PA, $P < 0.001$; FI/PA, $P = 0.02$). The dominant group, on the other hand, showed no significant change in SGR among moments and no weight loss was detected during the whole experimental period. Differences in growth between the hierarchical groups were found in the grouping period (PA – dominants/subordinates, $P = 0.003$).

The impact of pairing on fish growth can be better demonstrated by considering the variation in the specific growth rate (Δ SGR) between periods PA-EI and FI-PA for dominants and subordinates (Fig. 1B). According to the results obtained by the same statistical procedure used with SGR, fish from

both hierarchical ranks were negatively affected by pairing, a fact indicated by negative Δ SGR in PA-EI for both dominants and subordinates. When we consider Δ SGR between FI and PA, positive values were obtained for both dominants and subordinates, meaning that both groups increased their growth during the third period compared with the second. However, the magnitude of growth was different: subordinates showed significantly lower Δ SGR when compared to dominants in Δ PA-EI ($P = 0.04$) and only subordinates presented a significantly different value when comparing Δ PA-EI with Δ FI-PA ($P = 0.02$). No significant difference was found between dominants and subordinates in the mean of Δ FI-PA ($P = 0.1$). Such results indicate that fish of both hierarchical status lost weight in the pairing period, but subordinate lost significantly more than dominants. When fish are isolated again (FI) both dominant and subordinate fish increased the weight rate, but subordinates did it significantly more than dominants.

The effect of intermittent social stress on plasma cortisol and growth hormone levels are presented in Fig. 2. Only dominant fish had a significantly higher plasma cortisol levels after 8 days of pairing (PA - day 22) compared with the earlier isolation period (EI: day 1, $P = 0.01$; day 14, $P < 0.001$) (Fig. 2A). Two days after the stressor had been abolished (FI - day 24), fish of both hierarchical rank had cortisol levels significantly decreased compared with pairing (days 24 – 22, for dominants $P = 0.002$; for subordinates $P = 0.047$), with values similar to the early isolation period. However, after one week in post-pairing isolation (FI - day 29), the cortisol level of dominants was significantly higher compared with the entire early isolation period and final isolation day 24 (days 1, 14, 24, $P < 0.001$). Only on day 29 there was a

significant difference on cortisol levels between dominants and subordinates ($P = 0.03$), what means that fish of both hierarchical rank had a similar cortisol secretion throughout the experimental condition, but dominants showed a more intense response what reflected in significance in this group, but not in subordinates. Growth hormone levels (Fig. 2B - GH) showed the highest individual variability of all features measured. Friedman ANOVA followed by LSD post hoc test did not indicate any difference among time points for either dominant ($\chi^2 = 10.15$, $p = 0.12$) or subordinate animals ($\chi^2 = 18.12$, $p = 0.09$). Comparison between hierarchical ranks using Wilcoxon paired ranks test indicated that subordinates had a significant higher level of GH than dominants only on the second day after pairing (FI - day 24, $P = 0.03$), even though fish of both social rank showed a very low hormone concentration at this moment (less than 0.2 ng/ml) and it is the only moment that subordinate fish presented a higher level of GH than do dominants.

Energetic substrate levels are shown in Fig. 3. Although there were no differences in glucose means between dominants and subordinates in any condition, subordinate fish had a significant lower glucose level at the end of the pairing period (day 22) when compared with all the other days, except for day 1 (day 14, $P = 0.005$; day 24, $P = 0.003$; day 29, $P = 0.03$; day 35, $P = 0.02$); and dominants showed a significant increase in glucose level after the stressor was abolished (day 24) compared with the previous time point 2 days earlier (PA – day 22, $P = 0.03$) (Fig. 3A).

Both groups had a significantly higher level of protein 2 days after the stressor had been removed, when compared to levels on day 1 (day 24: dominants – $P = 0.02$; subordinates – $P = 0.004$). When amino acids levels are

considered, we observed that one week after the stressor had been removed (FI - day 29), subordinate animals showed significantly decreased amino acids levels compared with day 1 ($P = 0.004$) and day 24 ($P = 0.04$) followed by a significant increase on day 35 (compared with day 1, $P = 0.01$; days 14, 22, 24, 29, $P < 0.001$). When animals are compared according to the social rank, amino acid levels in dominant fish were significantly higher than in subordinates on days 29 and 35 (day 29, $P = 0.002$; day 35, $P = 0.04$), suggesting a different physiological adjustment between dominant and subordinate rainbow trout involving this metabolic substrate. According to Pearson correlation test results, no relationship between specific growth rate and hormones, specific growth rate and energetic substrate, or hormones and energetic substrate was detected.

Discussion

This study showed that intermittent chronic social exposure is a stressful condition for both subordinate and dominant rainbow trout and no habituation occurred after 8 days under this condition. However, the two hierarchical ranks do not present exactly the same range of responses and an enormous individual variation of the data among fish of the same hierarchical condition is evident.

It is presumed that under stress there is a reallocation of metabolic energy away from investment activities like growth toward activities required to restore homeostasis such as respiration, locomotion, hydromineral regulation, and tissue repair (Volpato and Fernandes, 1994; Alvarenga and Volpato, 1995; Barton, 2000). Another common idea in the literature is that in fish species where hierarchical ranks are well established, dominant fishes represent a stressor for subordinate ones, and subordinates usually show several

developmental consequences like decreases in growth and reproduction rates (Fernandes and Volpato, 1993; Wendelaar Bonga, 1997). Results obtained in this study are in agreement with the notion that subordinate fishes have their growth rate suppressed in the presence of dominants. However, the growth rate of dominants also decreased after the pairing period even though the rate remained positive. Thus, the magnitude of the response was different for each social rank. The significantly higher Δ SGR presented for subordinates in FI-PA, compared both with dominants at the same moment and among themselves at the moment PA-EI, suggests that when the stressor was abolished, subordinates grow at a higher rate than dominants. In terms of energy mobilisation, we can infer that during pairing, subordinate fish spent more energy than dominants on activities inherent to stress to the detriment of growth, but when the stressful condition is abolished, subordinates exhibit, for some period, a more intense investment in growth than dominants. Such a phenomenon is denominated compensatory growth and has been frequently described for fishes submitted to periods of low food availability (Metcalf and Monaghan, 2001). In this study, food was offered daily *ad libitum* when fish were separated by the partition but, as food intake was not quantified, it is not possible to know if the suppression and subsequent increase in growth rate was due to an exchange of food ingestion or of the energy available for growth. In any case, the growth responses are correlated with the effect of stress on fish metabolism, since competition for food did not occur.

The partition used to avoid direct contact between conspecifics did not suppress the stress condition for either dominant or subordinate fish. It needs to be tested whether that response was promoted by high aggression shown daily

when the partition was removed or resulted from another stimulus presented even with the partition, such as vision or chemicals. Although we did not quantify the agonistic profile when the partition was removed, we could observe that in most of the pairs fish were very aggressive, promoting subordinate death in 2 extra pairs during this period.

Cortisol is an important hormone involved in the chronic stress response in vertebrates (Chrousos et al., 1995; Csermely, 1998; Pickering and Pottinger, 1995; Slominski, 2000; Landys et al., 2006). In this study, dominant and subordinate animals presented a very similar response in plasma cortisol at all time points considered, except after pairing when dominants showed significant increase compared with subordinates. Although opposite results have been obtained with rainbow trout (Pottinger and Carrick, 2001; Sloman et al., 2001; Sloman et al., 2002; Fox et al., 1997), in which subordinates showed higher cortisol values than dominants, in all described studies fishes were in direct contact with each other. In the experimental procedure used herein, fish-to-fish contact was limited to 30 minutes/day and probably a more aggressive behaviour than usually displayed by dominants was involved with such cortisol response. The bimodal response of fish in the third period was unexpected. As blood samples were always collected in the afternoon, the differences cannot be ascribed to circadian fluctuations. The same type of response was obtained in rainbow trout following acute stress from manipulation (Farbridge, 1992). Those authors concluded that the cyclic pattern resulted from the rapid turnover of the hormone, followed by new secretion by the interrenal tissue, which perhaps occurred in the present experiment. Another interesting result obtained in our study was that one week after cessation of pairing, cortisol levels of

dominants were higher than those of subordinates, suggesting a different recovery time for the two hierarchical ranks.

The plasma GH values obtained in the present study were low in all groups at all time points considered and showed a large variation (high SEM) when compared with published data for the same specie under similar conditions (Kakizawa et al., 1995). The reports on circulating GH levels in stressed fish are inconsistent and difficult to interpret (Wendelaar Bonga, 1997). Plasma GH levels have been reported to increase after injection stress in goldfish (Cook and Peter, 1984) and to remain unaffected in chum salmon after handling stress (Wagner and Mckeown, 1986). The role of GH in social interaction is considered by some authors as indirect, by increasing swimming activities or inducing defense of a larger territory, thereby increasing the encounter rate between opponents (Jonsson and Bjornsson, 1994). According to Pérez-Sánchez and Le Bail (1999), a chronically high level of cortisol induces marked derangement of the GH axis; the predominant effect of acute stress may be the increase of GH release, whereas that of chronic stress could be a suppressive response. Farbridge and Leatherland (1992) suggested that in rainbow trout cortisol has a suppressive effect on GH, which possibly was the case in the present study.

Secondary stress indicators presented a very similar pattern of change between dominant and subordinate ranks. Although a higher value for free amino acids was obtained for dominants in the final phase of the recovery period (days 29 and 35) compared with subordinates, on the last day both dominants and subordinates had a higher level of amino acids than at any prior time, suggesting a role of these substrates in the increased growth activities.

According to McEwen and Wingfield (2003), allostatic load can be considered as the energy requirement of an animal resulting from the daily and seasonal routines plus the extra energy it needs to find a mate, breed, avoid predators, and so on. So, it contemplates the predictable events, like life history stages, and unpredictable situations like food deprivation, predators or social competitions. Social status may have an intense influence on allostatic load what is directly proportional to concentration of glucocorticoids presented by an animal (Goymann and Wingfield, 2004). Considering this idea, we can infer that the experimental condition used in our study promoted a dramatic increase in allostatic load of both social ranks, what reflected in reduced growth rate from dominants and subordinates, among others. When the pairing conditions was abolished, the physiological adjustment from dominants and subordinates were different according to variations on values of growth rate, cortisol and amino acids, for example, suggesting an different pattern of perception and /or adjustment to the new condition for dominants and subordinates.

Different recovery times (and different responses to intermittent social pairing) can be related to different stress conditions imposed, i. e., the stressor is not the same in both groups. To subordinates, the stressors are the presence of dominant fish, the bites and chases resultant from interactions when they were in direct contact with conspecifics plus the condition of submission. For dominants, the stressor is the presence of subordinates waiting to become dominant at the first opportunity and probably the impossibility of defining the dominance properly.

We can conclude that keeping rainbow trout in a chronic condition of intermittent social stress represents a stressful condition for both dominants and

subordinates. For subordinates the response seems to be more intense, but when the stress condition is abolished, subordinates tended to recover more quickly than dominants. The condition of daily short direct contacts between conspecifics does not seem to promote habituation in fish. In fact, the procedure used seems to represent a potent stressor for both members of the pair. We infer that in a more natural condition the interaction among fish must be an intermediary between what we used in this study, that did not permit to dominant keep your position properly and a continuous direct contact usually used in experiments involving social interaction, where subordinate is not able to emigrate away from the dominant fish.

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Fig. 1. In (A) specific growth rate (SGR, %) from dominant (unfilled bar, N = 8) and subordinate (filled bar, N = 8) rainbow trout in each experimental moment (EI = early isolation, PA = pairing, FI = final isolation). In (B) Δ SGR (%) between experimental moments PA – EI and FI – PA from dominant and subordinate fish. Each bar is the mean \pm SEM, N = 8. Asterisk (*) indicates significant difference between dominants and subordinates, # indicates difference between moments within the same hierarchical rank, P < 0.05.

Fig. 2. Plasma cortisol (A) and GH level (B) (mean \pm SEM, n = 8) from dominant and subordinate fish in each blood sample moment (1 and 14 (EI), 22 (PA), 24, 29 and 35 (FI) experimental days). Groups with the same lower-case character do not differ significantly from each other in different moments within the same hierarchical rank (repeated measure two way ANOVA followed by Tukey's post hoc test to cortisol or Friedman ANOVA followed by LCD post hoc test to GH); * indicates significant difference within the same moment between dominant and subordinate rainbow trout (the same statistical test indicated above to cortisol or Wilcoxon paired ranks test to GH, P < 0.05).

Fig. 3. Plasmatic energy substrates from dominant and subordinate fish (mean \pm SEM, $P < 0.05$) in each experimental moment (as presented in Fig. 2). A) plasma glucose. Significant main effect of days on glucose concentration was identified (repeated measures two-way ANOVA followed by Tukey's post hoc test, $F = 5.22$, $P < 0.001$), but no significant effect of hierarchical rank or interaction between day and hierarchical rank was identified, indicating that the pattern of plasmatic glucose presented by dominants and subordinates was the same. B) plasmatic protein concentration. Only significant main effect of days on protein concentration was identified ($F = 5.62$, $P < 0.001$) and C) plasma free amino acids. Significant main effects of both days and social status on plasmatic amino acids were identified ($F = 27.11$, $P < 0.001$ for the effect of day, $F = 5.13$, $P = 0.04$ for the effect of hierarchical rank). Lower-case letter shows comparison among different moments within the same hierarchical rank; * indicates significant difference between dominant and subordinate fish within the same period.

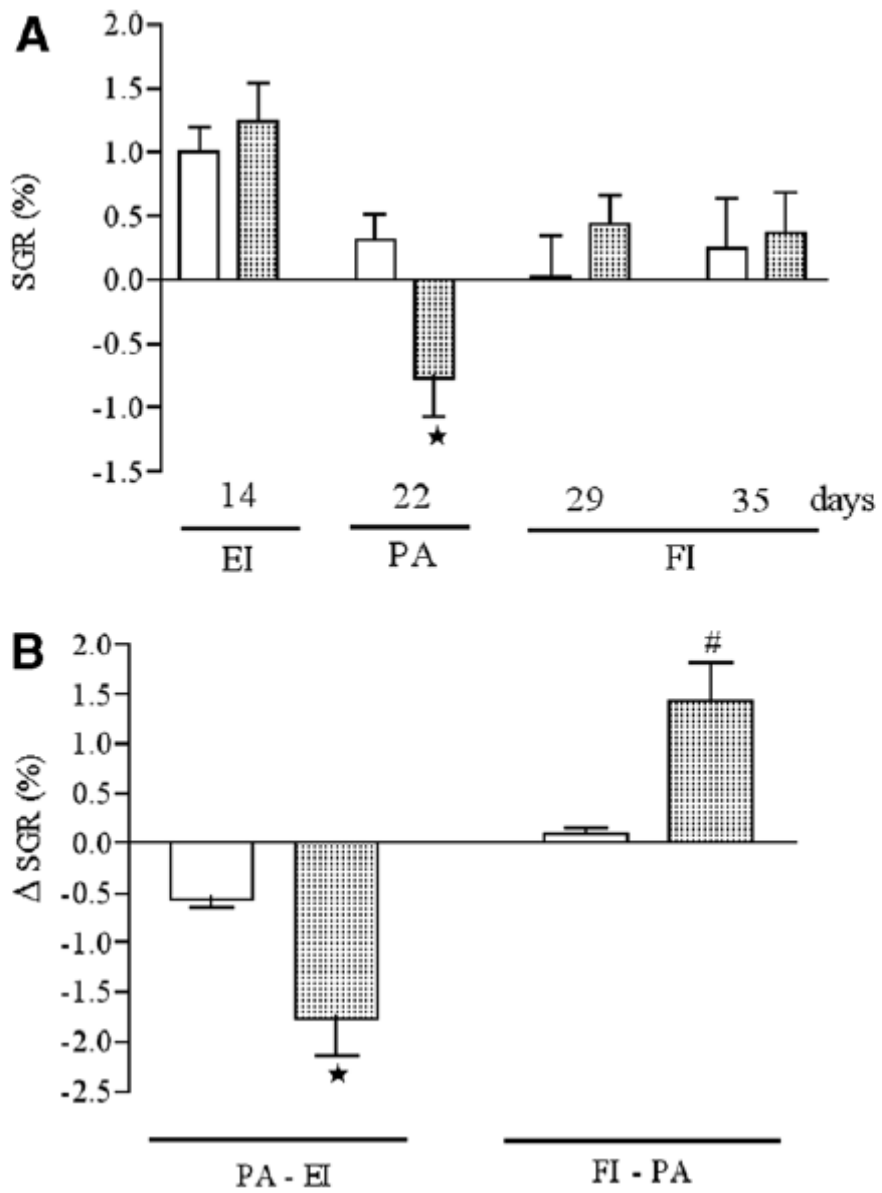


Figure 1.

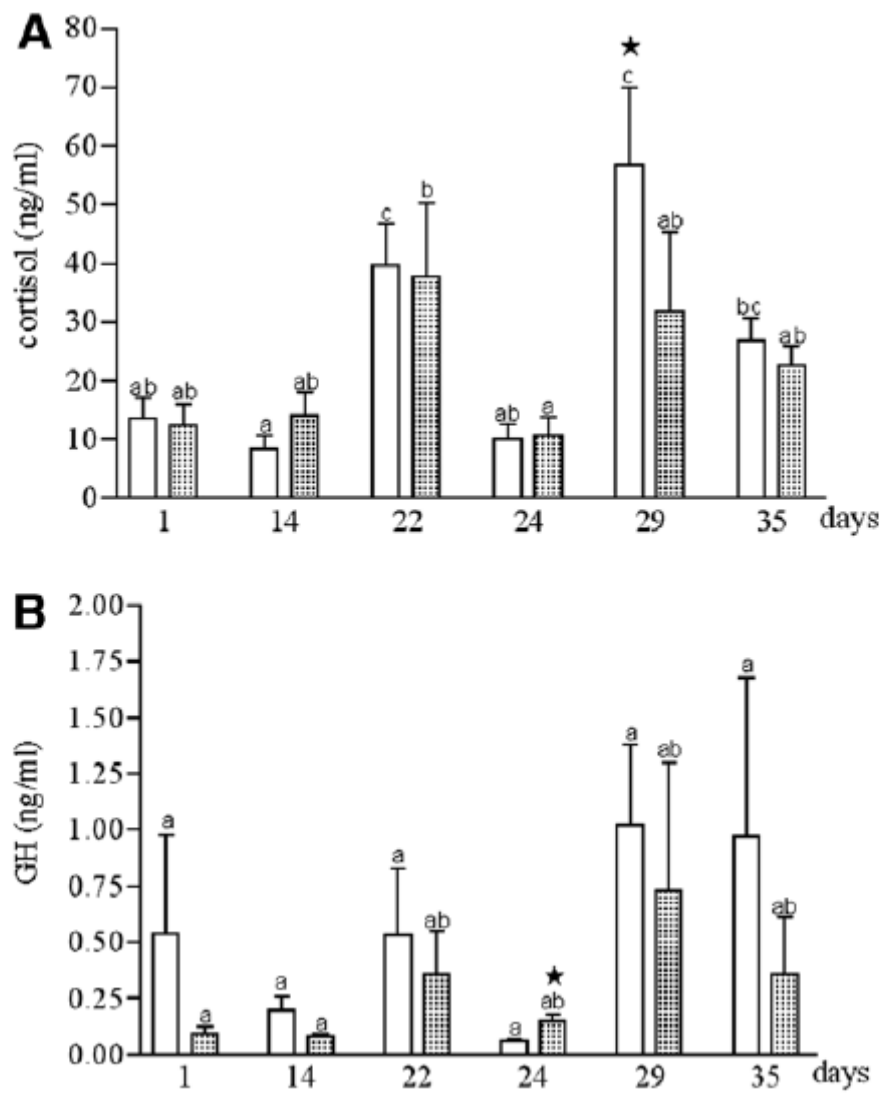


Figure 2.

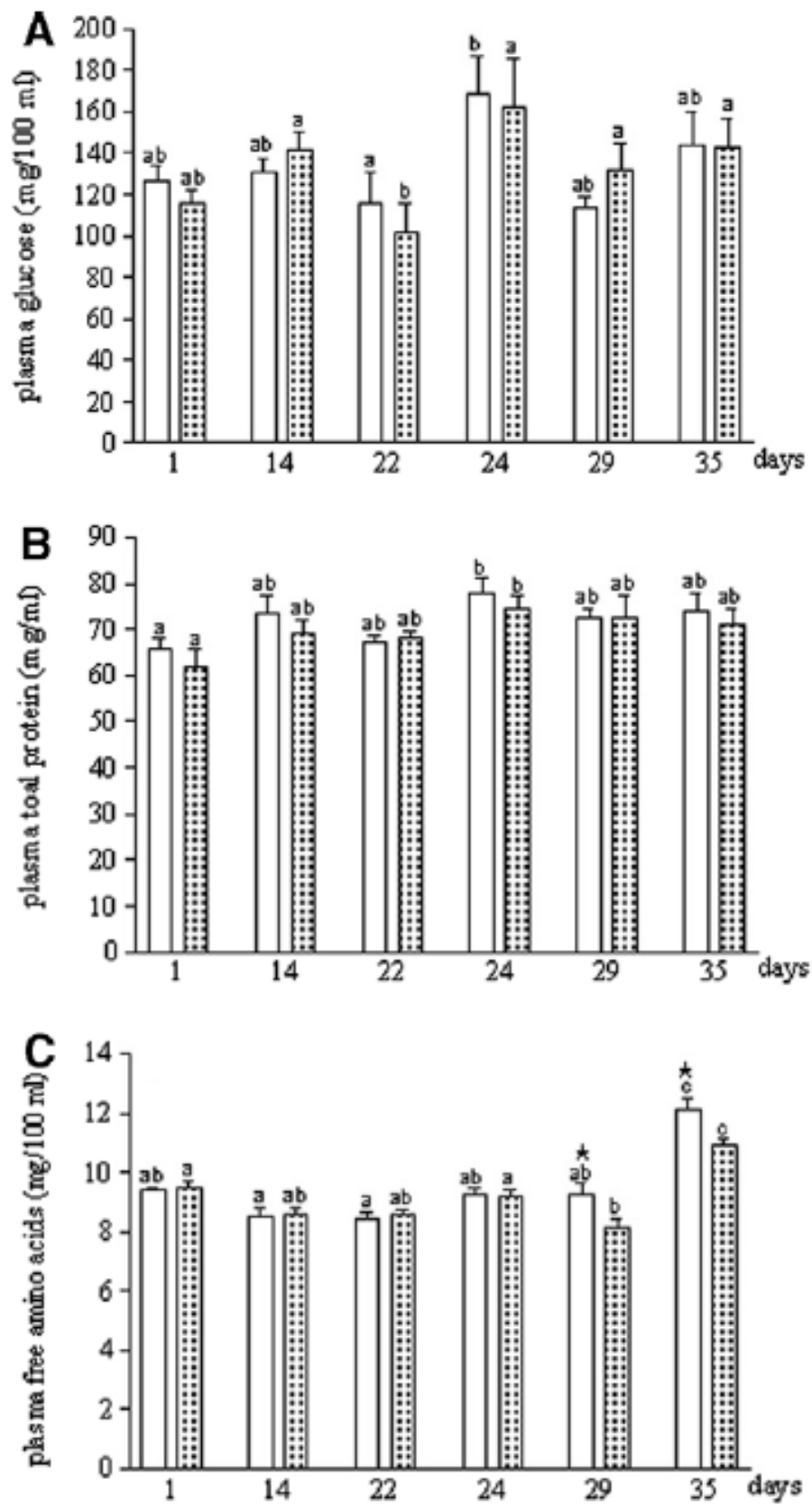


Figure 3