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EFFECT OF ANESTHETICS ON STRESS AND THE INNATE IMMUNE SYSTEM OF GILTHEAD SEABREAM *(SPARUS AURATA)*

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Key words: anesthetics, benzocaine, clove oil, innate immune system, *Sparus aurata*, stress

Abstract

Anesthesia may depress the immune system in mammals and fish. In the present work, two anesthetics used in aquaculture, clove oil (0.445 mM) and benzocaine (0.225 mM), were tested to observe their effects on the stress response and innate immune system activity of gilthead seabream (*Sparus aurata* L.). Results showed that both anesthetics induced increased blood glucose and serum cortisol levels. In addition, benzocaine depressed lysozyme activity, production of reactive oxygen species and pinocytosis activity. These were not depressed by clove oil, suggesting that clove oil is a safer anesthetic for seabream as it does not cause immunodepression in anesthetized fish.

Introduction

Anesthesia is a biological state induced by an external agent which results in the partial or complete loss of sensation or loss of voluntary neuromotor control through chemical or non-chemical means (Summerfelt and Smith, 1990). Anesthetics are frequently used in fishery studies and aquaculture to minimize stress response, preventing its negative impact on performance and reducing physical injury during handling procedures (Wedemeyer, 1997). While anesthesia benefits the fish by minimizing the impact of greater stressors, it is also inherently stressful and its effectiveness depends on the procedure used (Iwama et al., 1989). Severe anesthesia may induce a stress response in fish that can lead to immunodepression and increased susceptibility to disease (Iwama et al. 1989; Thomas and

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Robertson, 1991). Different anesthetic agents can cause diverse changes in physiological parameters.

Benzocaine is a common anesthesia, considered to be a local agent that blocks neural Na^+ channels, reducing transmission of nerve action potential (Carmichael, 1985). The problem in using benzocaine as an anesthetic is that it produces typical effects of stress such as hypoxia, hyperglycemia and increased blood lactate levels (Ross and Ross, 1984).

Clove oil has received favorable reviews as an alternative anesthetic for a variety of fish species (Keene et al., 1998; Cho and Heath, 2000). Eugenol (2-methoxy-4-2-(2-propenyl)-phenol), the active component of clove oil, is obtained from the buds, leaves and stem of the *Eugenia caryophyllus* plant. Clove oil has several advantages over other anesthetic agents in fishery research, assessment studies and aquaculture applications. It is an easily obtainable and inexpensive organic distillate, which is used as a food additive and possesses antifungal, antiviral, analgesic and antibacterial properties (Keene et al., 1998). Clove oil is organic, so no withdrawal period is required for fish intended for human consumption. Another advantage of clove oil is that it does not pose a chemical health hazard to the user. The only known disadvantage of clove oil is its photosensitivity.

The goal of this study was to examine the effect of two commonly used anesthetics, clove oil and benzocaine, on stress responses and innate immune system activity of the seabream (*Sparus aurata*).

Materials and Methods

Experimental fish and rearing conditions. Gilthead seabream (*Sparus aurata*), reared at the National Center for Mariculture (NCM) in Eilat, Israel, were used in this study. Prior to initiation of the experiment, sixty fish (220±15 g mean weight) were randomly distributed into each of four 900-liter flow-through tanks and acclimated for 21 days (40 ppt salinity, 1240 l/h flow rate, 5.6 mg/l aeration, 24±0.5°C, natural photoperiod). During the acclimation period, the fish were fed once a day at the rate of 1.2% body weight with commercial dry pellets (Matmor, M.P. Evtach, Israel).

Experimental design. Three groups of fifteen fish, each, were compared: control (no anesthetic), benzocaine-anesthetized (22.5 µl/l benzocaine) and clove oil-anesthetized (44.5 µl/l clove oil). The anesthetic was administered by dissolving it in 100% ethanol (1:20 for the benzocaine and 1:5 for the clove oil) and then in a small amount of sea water. The dissolved anesthetic was shaken and poured into the tank. The fish were exposed to the anesthesia until they reached a narcotic anesthesia state (loss of reflex reactivity, slow opercular movements). Blood samples were taken from the live fish, then they were killed and dissected.

Sampling. After exposure to the anesthetic, one ml of blood was collected from the caudal vein of each specimen using a 21-gauge needle and 1 ml syringe (in the stress examination, n = 15) within 5-7 minutes. Aliquots of 20 µl of the fresh blood samples were used for glucose analysis. The rest of the blood sample was allowed to clot at 4°C for 6 hours. Following centrifugation, the serum was removed and frozen at -20°C until the cortisol level and lysozyme activity were determined.

Head kidney leukocytes (in the immunological examination, n = 10) were isolated according to Steinhagen and Hespe (1997). The head kidney was dissected out by ventral incision and transferred to 5 ml RPMI-1640 medium supplement with 10 U/ml heparin (Sigma), 100 U/ml penicillin (Sigma), 0.1 g/l streptomycin (Sigma) and 5% fetal calf serum (Sigma). Cells were suspended by forcing fragments of the organ through a nylon mesh (100 µm). The head kidney suspension was washed three times, counted and adjusted to 40×10^6 in RPMI. Cell viability was greater than 95%, determined by the trypan blue exclusion test.

Stress indicators. The percent hematocrit was determined by centrifuging the total blood in heparinized microhematocrit tubes and calculating the percent of erythrocytes. Samples of 0.02 ml fresh blood were used to measure glucose levels by the “One Touch Basic” glucose meter (Shnitman, 1995). Serum cortisol levels were determined with a tritium H^3 radio

immunoassay (RIA). Cortisol in the samples competes with the radioactive labeled cortisol to bind with anti-cortisol antibody. Radioactivity levels were measured in a Beta-counter (Redding et al., 1984).

Lysozyme activity. The lysozyme activity assay was performed according to Ellis (1990). Analysis of the lysozyme level was based upon the lyses of the lysozyme-sensitive gram-positive bacterium *Micrococcus lysodeikiticus* (Sigma). The bacteria lysis was measured turbimetrically (ELISA reader, Spectra 2000, SLT, Germany) at 492 nm, 0.5 and 4.5 min after the start of the reaction. The reaction results were calculated as lysozyme activity units per 1 ml fish serum.

Production of reactive oxygen species (ROS). The ROS production assay was done according to Secombes (1990). Three replicates of leukocytes from each fish were incubated for two hours in 96 microtiter plates with nitroblue tetrazelium (NBT; Sigma) and phorobol myristate acetate (PMA; Sigma). During the reaction, soluble yellow NBT is reduced by O_2 to insoluble blue formezan. The amount of blue formezan was measured after it was dissolved with KOH (Sigma) and DMSO (Sigma). The optical density (OD) was read with a spectrophotometer (ELISA reader, Spectra 2000, SLT, Germany) at 620 nm and 405 nm.

Pinocytosis activity. The pinocytosis assay was done according to Mathews et al. (1990). The leukocytes were incubated in microtiter plates for two hours together with neutral red (Sigma), washed twice and dissolved with acid alcohol. The OD of the neutral red released into the supernatant was measured spectrophotometrically at 492 nm (ELISA reader, Spectra 2000, SLT, Germany)

Statistical analysis. All assays were performed in triplicate and the mean±standard error (SEM) was calculated for each group (no. fish = 10 for immune parameters and 15 for stress parameters). A one-way analysis of variance (ANOVA) with Newman-Keuls multiple range test was performed to discover differences between means. Differences were considered statistically significant when $p<0.05$.

Results

Anesthesia. In preliminary experiments (data not shown), both benzocaine and clove oil (at the same dosage) caused rapid anesthesia in the fish, which lost sensation and equilibrium no more than 8 minutes after the anesthesia was induced. The fish recovered from the anesthesia no more than 90 seconds after they were transferred to a new tank. No mortality was observed in either group. In the control group, three fish died within 72 hours after sampling.

Blood glucose concentration. The blood glucose concentration of the anesthetized fish was significantly higher than that of the control fish with both anesthetics (Fig. 1). There was no significant difference in glucose concentration between fish anesthetized with clove oil and those anesthetized with benzocaine.

Hematocrit. The hematocrit level of the control fish was significantly higher than that of the anesthetized fish (Fig. 2). There was no significant difference in hematocrit levels of fish anesthetized with clove oil or benzocaine.

Serum cortisol concentration. Serum cortisol levels of the anesthetized fish were significantly higher than that of the control fish with both anesthetics (Fig. 3). The cortisol level of fish anesthetized with benzocaine was significantly higher than that of fish anesthetized with clove oil.

Serum lysozyme activity. The serum lysozyme activity (measured in unit/ml/min) was significantly reduced by benzocaine (Fig. 4). There was no significant difference between the control and the clove oil anesthetized fish.

Respiratory burst activity. The respiratory burst activity of seabream head-kidney leucocytes was significantly reduced by benzocaine (Fig. 5). There was no significant difference between the control and the clove oil anesthetized fish.

Pinocytosis activity. The pinocytosis activity of seabream head-kidney leucocytes was significantly reduced by benzocaine (Fig. 6). There was no significant difference between the control and the clove oil anesthetized fish.

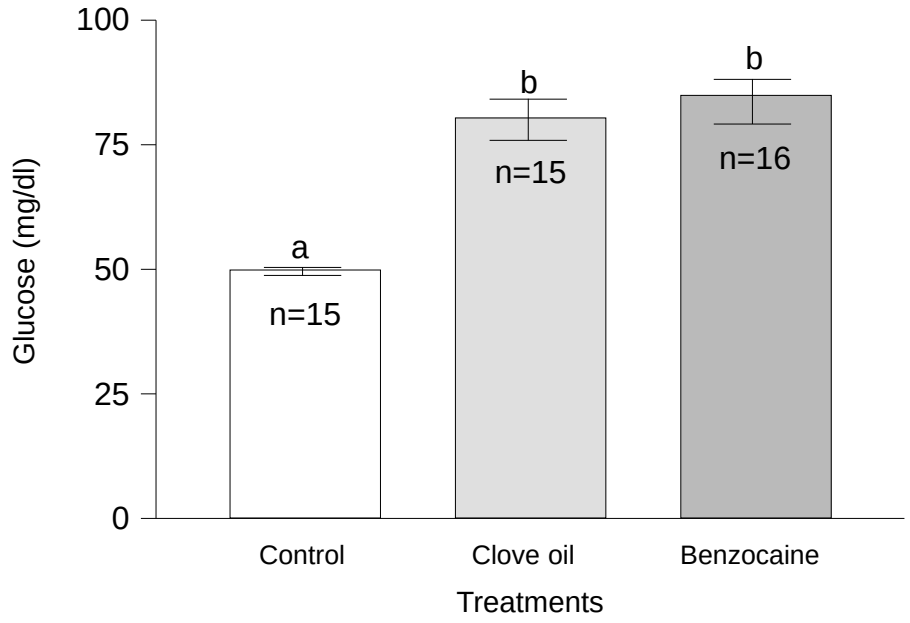

Fig. 1. Blood glucose levels of anesthetized and control gilthead seabream. Data represent means±SD. Different letters denote a significant difference between groups.

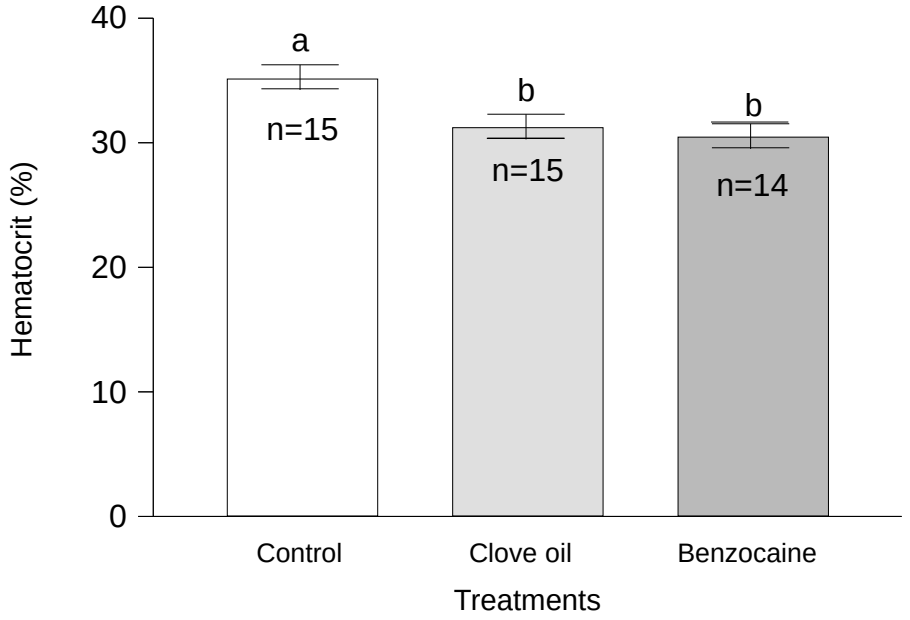

Fig. 2. Hematocrit levels of anesthetized and control gilthead seabream. Data represent means±SD. Different letters denote a significant difference between groups.

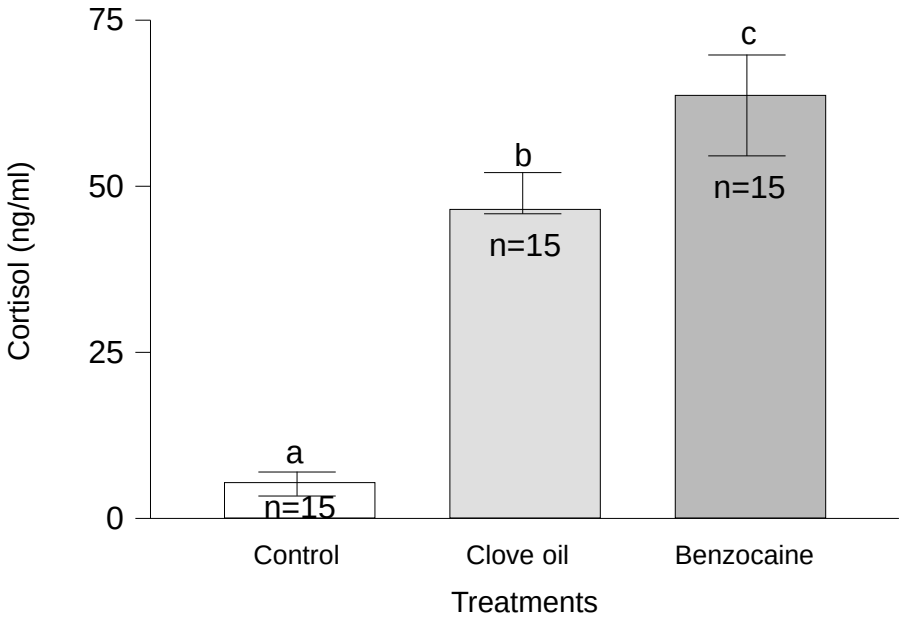

Fig. 3. Serum cortisol concentration of anesthetized and control gilthead seabream. Data represent means±SD. Different letters denote a significant difference between groups.

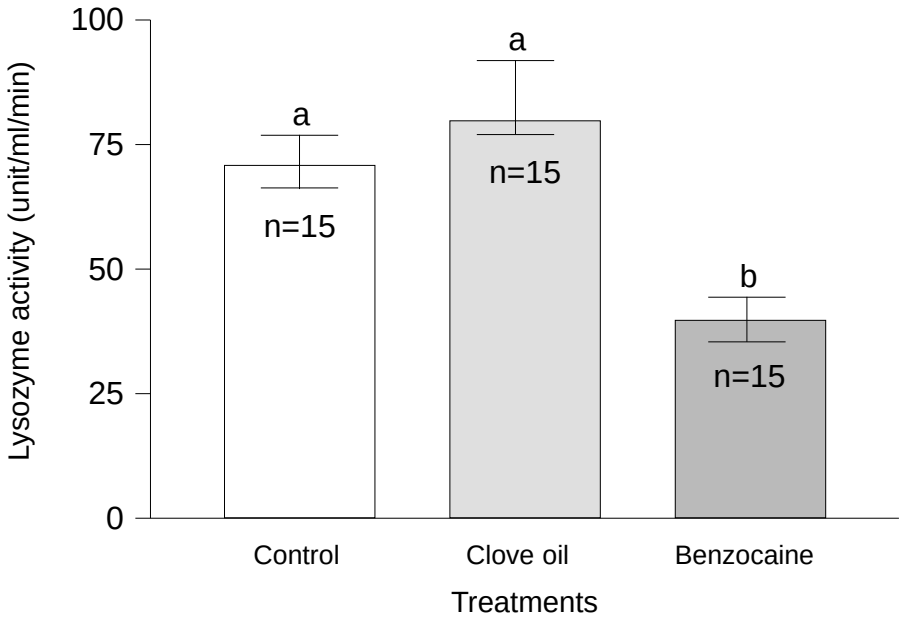

Fig. 4. Serum lysozyme activity of anesthetized and control gilthead seabream. Data represent means±SD. Different letters denote a significant difference between groups.

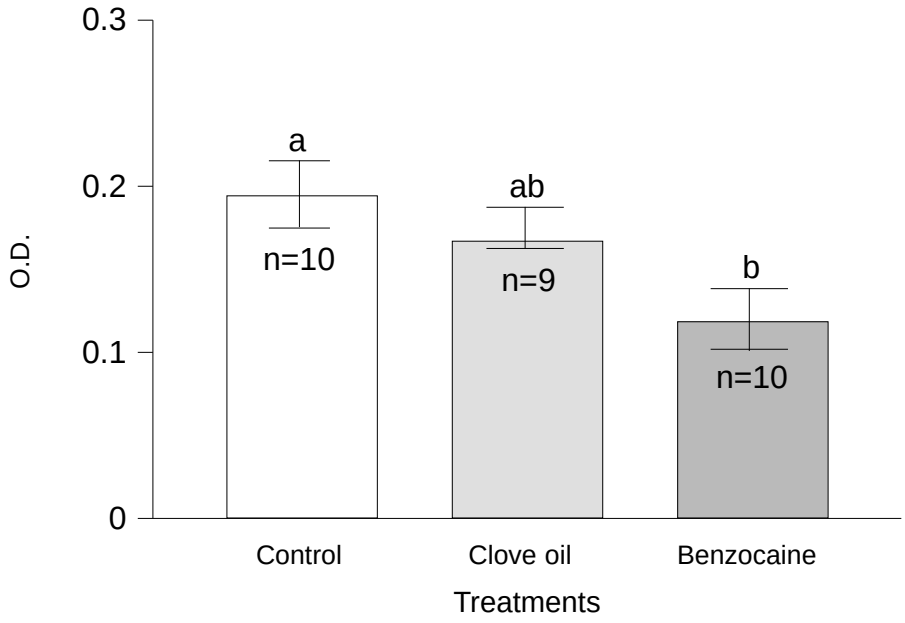

Fig. 5. Respiratory burst of head-kidney leukocytes from anesthetized and control gilthead seabream. Data represent means±SD. Different letters denote a significant difference between groups.

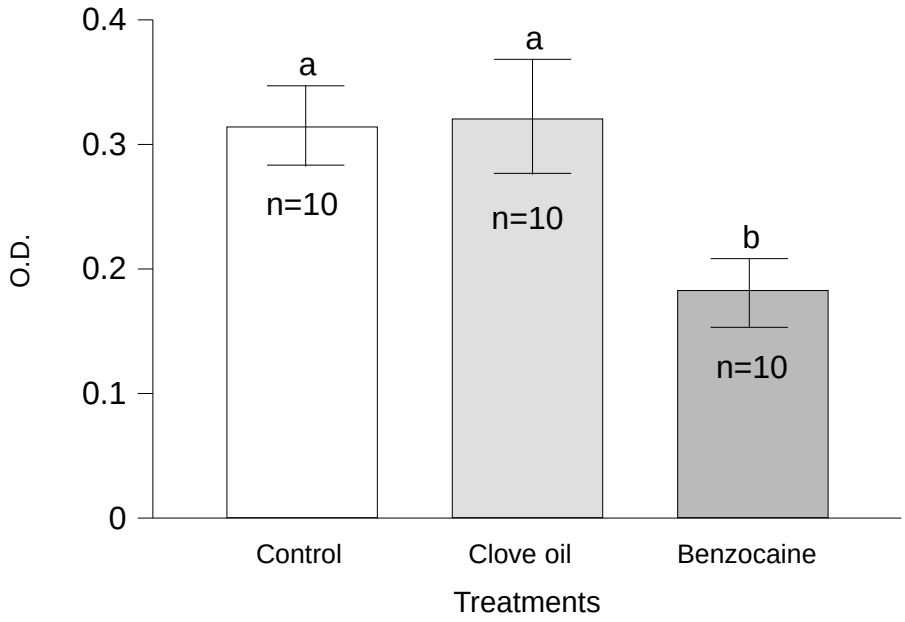

Fig. 6. Pinocytosis activity of head-kidney leukocytes from anesthetized and control gilthead seabream. Data represent means±SD. Different letters denote a significant difference between groups.

Discussion

Previous studies have shown that anesthetics can reduce fish stress responses when suitably administrated, preventing the negative impact of stress on fish performance and reducing physical injury during handling (Robertson et al., 1988; Wedemeyer, 1997). Indeed, in the present study there was no mortality in the anesthetized fish after blood sampling in comparison with 20% mortality in the control fish. On the other hand, some anesthetics may induce a stress response in fish when exposure time is too long (Iwama et al., 1989; Thomas and Robertson, 1991). Blood glucose and serum cortisol concentrations are commonly used as stress indicators in fish studies because they have proven to be reliable endocrine and secondary indicators for many stressors to fish and are easily measurable parameters (Schreck, 1981). Several studies demonstrated the influence of anesthetics on the magnitude of corticosteroid and hyperglycemic responses of fish to stress (Iwama et al., 1989; Ortuno et al., 2002a).

In the present study, benzocaine and clove oil induced a stress response in the fish, indicated by the increase in blood glucose level and serum cortisol concentration. The rise of serum cortisol in this study is coincident with the increase in blood glucose. This well-known pattern of hyperglycemia after stress has been shown to result from catecholamine and corticosteroids released into the blood and has been reported in other research (Anderson et al., 1991; Ortuno et al., 2002b). The glucose value and serum cortisol concentration of the control fish in this study were typical for this species as well as other sparids (Rotllant and Tort, 1997; Ortuno et al., 2002b). Presumably, the intensity of the sampling stress was below the threshold required to induce corticosteroid and glucose stress responses (Thomas and Robertson, 1991). Increasing the duration of the sampling time to 15-20 minutes elicited stress responses in gilthead seabream and several-fold increases in serum cortisol and blood glucose levels in our preliminary experiment (data not shown). In the present study, the anesthesia did not cause an elevation of the percent hematocrit. Similar results were reported in another study using a similar concentration of clove oil anesthesia in seabream (Tort et al., 2002).

Anesthetic agents are known to have immunodepression effects by depressing phagocytic functions such as leucocyte recruitment, attachment, chemotactic motility, engulfment and intercellular killing in mammals and fish (Bardosi et al., 1992; Ortuno et al., 2002a; Puig et al., 2002). Several researchers have showed that lysozyme and phagocytic activity can be used as immune indicators (Demers and Bayne, 1997; Ortuno et al., 2002c). Other studies have shown a correlation between an elevated plasma level of cortisol, immunodepression and increased susceptibility to disease in cultured fish (Thomas and Lewis, 1987; Maula et al., 1989). A study on mice showed that stress induced a rise in plasma corticosterone and inotropes and might be involved in modifying the distribution of neutrophils and lymphocytes (Puig et al., 2002). Another fish study found a correlation between modification of the glucose concentration and complement activity. This study also found a correlation between modification of the cortisol level and phagocytosis. These studies suggest that adrenaline has a stronger effect on the complement system and cortisol has a stronger effect on the phagocytic function (Ortuno et al., 2002c). On the other hand, another study suggested that the immunodepression caused by the anesthesia is not caused by stress signals, but by direct interaction between the anesthetic agent and immune components, or through the nervous system (Ortuno et al., 2002a). In the present study, there was no immunodepressive effect of anesthesia with clove oil. On the other hand, both humoral and cellular immune responses were significantly depressed by benzocaine. Our results are consistent with those reported for seabream by Ortuno et al. (2002a).

Our research showed that anesthesia may affect fish stress responses and therefore might be unsuitable for some research purposes. In aquaculture, the use of clove oil as an anesthetic agent is preferred to the use of

benzocaine because it does not pose a chemical health hazard to people who handle the fish or to consumers nor does it cause any significant immunodepression effect on the fish.

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ERRATUM

In the article "Defining energy and protein requirements of gilthead seabream *(Sparus aurata)* to optimize feeds and feeding regimes" by Ingrid Lupatsch, George Wm. Kissil and David Sklan, published in the December 2003 issue 55(4), the recommended supplies of digestible energy for gilthead seabream shown in Table 3 refer to temperatures of 20°C and 26°C as indicated in the text and column headings, and not as in the table caption. We regret this editorial error.