The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement

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Abstract

We investigated short-term effects (up to 24 h) of air exposure and confinement, and long-term effects (up to 11 days) of confinement, to elucidate signalling pathways in the stress response of gilthead sea bream *Sparus aurata* L. Plasma glucose and lactate were taken as indicators of sympathetic activation, and α-melanocyte stimulating hormone (α-MSH), adrenocorticotrophic hormone (ACTH) and cortisol as indicators of activation of the brain–pituitary–interrenal (BPI) axis. Air exposure for 3 min resulted, within 30 min, in an increase in plasma concentrations of cortisol, α-MSH, glucose, lactate, osmolality and plasma Na, Cl and Mg. Plasma ACTH and β-endorphin and plasma K, Ca and P did not change. We conclude that air exposure mainly activates the brain–sympathetic–chromaffin cell (BSC) axis. In fish confined at a density of 70 kg/m^3 (compared with 4 kg/m^3 in controls), cortisol, ACTH and α-MSH increased within 1 h, indicating activation of the BPI axis. Plasma glucose, Na, Cl and Mg increased with an 8 h delay compared with the response to air exposure. No changes in plasma lactate, osmolality, K, Ca and P were observed. Long-term confinement induced a biphasic cortisol response with peaks at 1 h and at 2 and 3 days. A gradual increase in plasma β-endorphin concentrations peaked at 7 days; the concentration of α-MSH increased rapidly within 1 h and then declined to control values 4 days after the onset of confinement. No changes in ACTH were detected. Our data provide evidence that a stressor-specific activation of the BSC and BPI axes may occur in *Sparus aurata*.
Journal of Endocrinology (1999) **163,** 149–157

Introduction

In aquaculture, fish experience stressors such as handling and confinement, which disturb homeostatic equilibria. Reponses to stress-related disturbances in fish are often characterized as primary, secondary, and tertiary (Barton & Iwama 1991). A primary response is the activation of brain centres that eventually results in the release of cortisol from the steroid-producing cells and of catecholamines from the chromaffin cells of the head kidney. Secondary responses are defined as the subsequent actions and effects of these hormones at blood and tissue level, and may include a disturbance of the hydromineral and metabolic balance. Tertiary responses, exemplified by inhibited growth, hampered reproduction and immunosuppression, concern the performance of the organism.

For fresh-water fish, the primary stress response has been studied extensively. The principal messenger systems involved are the brain–sympathetic–chromaffin cell (BSC) axis and the brain–pituitary–interrenal (BPI) axis (Wendelaar Bonga 1997). A direct activation of chromaffin cells by sympathetic nerve endings has been demonstrated in fish (Gfell *et al.* 1997). Stressors such as handling and hypoxia have frequently been shown to result in a rapid increase in plasma glucose and lactate, and this has been associated with the activation of the BSC axis and the release of catecholamines by the chromaffin cells in many teleosts (Barton & Iwama 1991, McDonald & Milligan 1992, Randall & Perry 1992). Therefore, glucose and lactate can be considered as indicators of sympathetic activation during stress. Activation of the BPI axis results in the release of cortisol by the interrenal cells of the head kidney. Adrenocorticotrophic hormone (ACTH) is considered the most potent pituitary corticotroph in fish and in terrestial animals, but in tilapia (*Oreochromis mossambicus*) another pro-opiomelanocortin (POMC)-derived peptide, *viz*. α-melanocyte stimulating hormone (α-MSH), also has corticotrophic actions (Lamers *et al.* 1992). Moreover, acetylated endorphins secreted by the α-MSH cells have been suggested to potentiate the corticotrophic activity of α-MSH (Balm *et al.* 1995). Differential activation of the ACTH and the MSH cells have been reported in fish. In

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salmonids, handling and confinement activate the BPI axis and increase plasma concentrations of ACTH, α-MSH and β-endorphins (Sumpter *et al.* 1985, 1986). In these fish, plasma α-MSH concentrations were increased only when handling and confinement were combined with a thermal shock. Comparable results were obtained for *Oreochromis mossambicus*. In these fish, netting increased plasma cortisol concentrations, but did not affect the plasma ACTH concentration, whereas the increase in cortisol after netting and confinement was associated with increased ACTH (Balm *et al.* 1994). However, there is still a lack of knowledge about the interplay between the two signalling pathways.

Compared with fresh-water fish, little is known about responses of sea-water fish to stressors. Most studies on sea-water species deal with transfer of fish from sea water to brackish or fresh water. *Sparus aurata* from full-strength sea water adapt in a very short period to brackish water. As *Sparus aurata* can not only hyporegulate in sea water, but also hyperregulate in brackish water, the gilthead sea bream is considered to be a truly euryhaline fish (Mancera *et al.* 1993, 1995, Altimiras *et al.* 1994). For the gilthead sea bream, it has been demonstrated that cortisol has mineralocorticoid actions that facilitate adaptation to changes in ambient salinity (Mancera *et al.* 1994, Altimiras *et al.* 1994). In the same fish, crowding and repeated daily acute stress result in immunosuppression (Sunyer *et al.* 1995, Tort *et al.* 1996). In the red gurnard (*Chelidonichthys kumu*), capture and confinement negatively affect reproduction (Clearwater & Pankhurst 1997). Although there are several reports on cortisol in stressed sea-water fish, the pituitary messengers involved in these responses have so far received little attention. However, recently, a stressor-specific activation of the endorphin system has been demonstrated. An increase in plasma *N*-acetyl β-endorphin was found in sea bream stressed by confinement and crowding, whereas confinement alone had no effect on the plasma acetyl endorphin concentration (Mosconi *et al.* 1998).

In the present study, the endocrine responses to air exposure and confinement were investigated in the gilthead sea bream (*Sparus aurata* L.) in full-strength sea water. The experiments were designed to investigate the principal messenger systems activated upon exposure to these stressors. Differential effects of air exposure and confinement on the primary (hormonal), and secondary (hydromineral and metabolic) responses were found.

Materials and Methods

Fish

Immature male gilthead sea bream (*Sparus aurata* L., hereafter called 'sea bream'), weighing 60–100 g, were obtained from an experimental fish culture centre (El Toruño, Pemares, El Puerto de Santa Maria, Cádiz). During the experiments (September–November 1997), the fish were kept under environmental conditions of photoperiod and water temperature (18–22 °C). For each experiment, 200 fish were kept in well-aerated 5000 l stock-tanks at a density of 4 kg/m^3. The water was continuously refreshed (250 l/h) and supplied with air through air stones. Fish were fed twice a day (0900 h and 1700 h) with 1% body weight commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain).

Experimental procedure

Fish, 12–16 per group and one group for each time point, were transferred from their stock-tank to grey cylindrical experimental tanks (volume 500 l; diameter of tank 0·85 m), each containing a plastified iron wire-net cage with a total volume of 250 l (inner diameter of cage 0·60 m) to obtain a fish density of 4 kg/m^3; the fish were allowed to acclimate to the experimental tank for 6 days. Feeding was stopped 24 h before the experiments. No mortality was observed in any group during the experiments. To minimize confounding effects of tank-related influences, control and experimental groups were sampled from randomly chosen tanks at randomly chosen time points during each experiment.

Daily variation at sampling time points Fish (n=8 per group) were sampled at 0900, 1100, 1300, 1700 and 1900 h and at 0900 h the next day. They were anaesthetized for 1 min by taking the cage out of the tank and transferring the fish to a bucket containing 0·1% 2-phenoxyethanol (Sigma). Once anaesthetized (always within 1 min), the fish were taken out of the bucket. One millilitre of blood was taken from the caudal vessels using a syringe containing 35 μl 2% Na_2-EDTA (Sigma) and 25 μl Trasylol (equivalent to 250 kallikrein inhibiting units, Bayer). All blood samples were taken within 5 min after capture (n=8 per group). Plasma was separated from cells by centrifugation for 20 min at 3000 r.p.m. and was stored at -20 °C until required for further analyses.

Experiment I: air exposure Fish were exposed to air for 3 min, by lifting the wire-net cage out of the tank, after which the cages were put back into the tanks. Fish (n=8 per group) were sampled at 0 (without air exposure) and at 0·5, 1, 2, 8, 12 and 24 h after air exposure, as described above. This experiment was repeated three times for t=0 and t=0·5 h.

Experiment II: 24 h confinement Fish were confined up to 24 h by lifting the wire-net cage in the tank (water depth about 10 cm) to increase the stocking density from 4 to 70 kg/m^3. This treatment is hereafter referred to as confinement. Fish (n=8 per group) were sampled at 0 (before confinement) and at 0·5, 1, 2, 4, 8, 12 and 24 h during confinement, as described under 'Daily variation'.

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Experiment IIIa: prolonged confinement Fish were confined up to 11 days, by lifting the wire-net cage in the tank to increase the density to 70 kg/m^3. Fish (n=8 per group) were sampled at 0 (before confinement) and at 1, 2, 3, 4, 7 and 11 days during confinement, as described under 'Daily variation'.

Experiment IIIb: the role of fasting on prolonged confinement Fish were fasted for 7 days at a density of 4 or 70 kg/m^3 and sampled as described under 'Daily variation'.

Analyses

Osmolality and plasma minerals Osmolality was measured with a freezing-point depression osmometer (Roebling osmometer type 4B). Plasma Na and K concentrations were determined by flame photometry (Radiometer Copenhagen FLM3 flame photometer); the Cl concentration was determined spectrophotometrically with the ferrothiocyanate method (on 250-fold diluted plasma). Plasma total Ca, Mg and P concentrations were measured by ICP-AES (100-fold diluted plasma; Spectro analytical instruments, Spectroflame).

Hormone RIAs The plasma cortisol concentration was measured with a highly specific antibody for cortisol (ImmuChem Cortisol ^{125}I RIA kit, ICN Biomedicals, Inc., Costa Mesa, CA, USA) in 25 µl plasma. The radioactivity was quantified using a Cobra II γ-counter (Packard Intruments Company). Cross-reactivity with cortisone was less than 1·5%. The intra-assay variation was 7·0% (n=20), and the interassay variation 7·9% (n=15). The RIA for α-MSH was based on an antibody described by Vaudry *et al.* (1978), and was used in a final dilution of 1 : 60 000. The cross-reactivity of this antiserum with desmono- and di-acetylated α-MSH is 100%. Immunocomplexes were precipitated with 15% (w/v) polyethylene glycol and 2·4% (w/v) ovalbumin as described previously (van Zoest *et al.* 1989). ACTH was measured in a RIA described by Balm *et al.* (1994) for tilapia, using an antibody raised in rabbit against human $ACTH_{1–24}$ (Dores *et al.* 1993). Immunocomplexes were collected by precipitation with a sheep anti-rabbit second antibody and 7·5% (w/v) polyethylene glycol. Pituitary homogenates of sea bream prepared in 0·01 M HCl and diluted in RIA assay buffer displaced radiolabelled ACTH from the antibody in parallel with dilutions of the standards used. Cross-reactivity with α-MSH was negligible. For the RIA of β-endorphins, an antiserum recognizing N-terminally acetylated endorphins was used (Takahashi *et al.* 1984). The antiserum has full cross-reactivity with acetylated forms of mammalian β-endorphins, whereas cross-reactivity with non-acetylated endorphins is less than 0·1% (Dores *et al.* 1991). For RIA, the antibody was used in a final dilution of 1 : 100 000. Immunocomplexes were precipitated with 15% (w/v) polyethylene glycol and 2·4% (w/v) ovalbumin.

Plasma glucose, lactate and protein Plasma glucose and lactate were measured using commercial kits from Sigma, St Louis, USA (Iwama *et al.* 1989). Plasma protein was measured using the bicinchoninic acid (BCA) method (Smith *et al.* 1985) with a BCA protein kit (Pierce, Rockford, USA).

Statistical analysis

In all experiments, differences among groups were assessed by means of one-way analysis of variance (ANOVA). Subsequently, significance of differences between mean values was tested with the Dunett's multiple comparison test or the Kruskal–Wallis rank sum test. Where necessary to improve homogeneity of variance, appropriate transformations of the data were carried out. Statistical significance was accepted at $P<0·05$. Values (n=8 for all groups) are depicted as means ± standard error of the mean (S.E.M.).

Results

We first measured plasma concentrations of cortisol, glucose, lactate, Na, K, Cl, Ca, Mg, P and osmolality at the time points of sampling during the experiments to check for daily variations in these values. Plasma cortisol concentrations were slightly increased around feeding time, *viz.* at 0900 and 1700 h. These increases in plasma cortisol disappeared when feeding was stopped 24 h before sampling. No variations at sampling time points were found for plasma glucose, lactate, osmolality and mineral concentrations (results not shown).

Differences in behaviour were observed in fish exposed to the different stressors. During 3 min of air exposure, the fish showed an escape reaction (floundering) during the first 1 min of air exposure, followed by a quiet period of about 1 min before they started floundering again. Fish stressed by confinement were quietly drifting (swimming was hampered by the low water level) to the water inlet and air inlets, probably to supply the gills with freshly oxygenated sea water.

The short-term hormonal responses of sea bream to air exposure (experiment I) and confinement (experiment II) are shown in Fig. 1. Plasma cortisol concentrations in air-exposed fish increased more than 50-fold within 30 min (from 25 nM at t=0 to 1350 nM at t=30 min). Confinement increased plasma cortisol concentrations about eightfold within 1 h (from 25 nM at t=0 to 202 nM at t=1 h; Fig. 1*a*). After 2 h, plasma cortisol concentrations had returned to control values in both groups, and they remained so for another 22 h. In air-exposed fish, plasma ACTH concentrations decreased to about 30% of control

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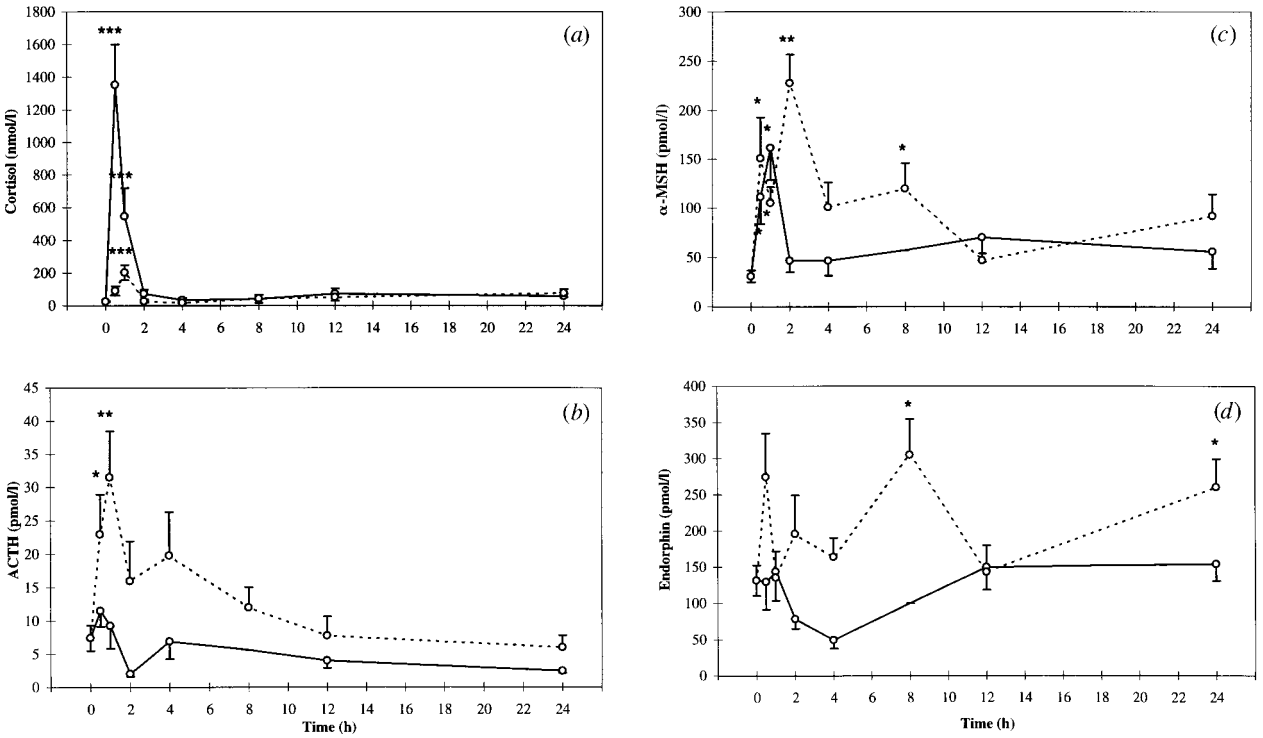

Figure 1 Changes in plasma cortisol (a), ACTH (*b*), α-MSH (*c*) and *N*-acetyl β-endorphin (*d*) concentrations of sea bream subjected to 3 min air exposure (solid line), or during confinement (dotted line). **P*<0·05, ***P*<0·01, ****P*<0·001, significant differences compared with control at *t*=0 (*n*=8; means ± S.E.M.).

after 2 h. Plasma ACTH had increased 30 min after onset of confinement (Fig. 1*b*) and remained increased for almost 4 h. Maximum plasma ACTH concentrations, a more than fourfold increase (from 7 pM at *t*=0 to 32 pM at *t*=1 h), were found 1 h after the onset of confinement. Plasma α-MSH concentrations had increased fivefold at 1 h for air-exposed fish, and more than sevenfold at 2 h for the confined group (Fig. 1*c*). Plasma *N*-acetyl β-endorphin concentrations had decreased by 37% of the control value at 4 h in air-exposed fish, but were more than doubled at 8 h and 24 h in fish experiencing confinement (Fig. 1*d*). To overcome confounding effects of tank-related influences, this air-exposure experiment was carried out three more times. Thirty minutes after air exposure, plasma ACTH concentrations never exceeded 17 pM. Furthermore, sampling at several time points within 30 min after air exposure did not reveal increased plasma ACTH concentrations.

Long-term confinement (experiment IIIa) induced a cortisol response on days 2 and 3 after the onset of the confinement (Fig. 2*a*). An almost 10-fold increase in plasma cortisol was measured at day 3. This increase in plasma cortisol was accompanied by an increase in plasma glucose up to 3 days after onset of the confinement. After 4 days, cortisol and glucose concentrations had returned to control values. No changes in plasma ACTH concentrations were seen throughout this long-term confinement experiment (Fig. 2*b*). Prolonged confinement evoked a threefold increase of α-MSH, 1 day after the onset of the confinement. After 3 days, α-MSH concentrations declined again to control values (Fig. 2*b*). A gradual increase in plasma β-endorphin was measured in the long-term confinement experiment, with a more than fivefold increase over controls at day 7. After 11 days of confinement, fish had plasma β-endorphin concentrations that were still increased. Both fasting and confinement (experiment IIIb) slightly increased plasma β-endorphin concentrations (Fig. 2*c*). In fasted and confined fish, plasma β-endorphin was significantly increased compared with controls.

Figure 3 shows the effects of air exposure (experiment I) and confinement (experiment II) on glucose and lactate. Plasma glucose increased in air-exposed fish within 30 min by about 75% compared with unstressed fish (Fig. 3*a*). After 12 h, glucose had returned to control concentrations. During confinement, plasma glucose increased 12% after 2 h, and remained increased for the duration of the confinement (Fig. 3*a*). A threefold increase in plasma lactate concentrations was measured 30 min after air exposure (Fig. 3*b*). Lactate concentrations had declined to control values 2 h after exposure. In contrast, confinement did not influence plasma lactate concentrations. The

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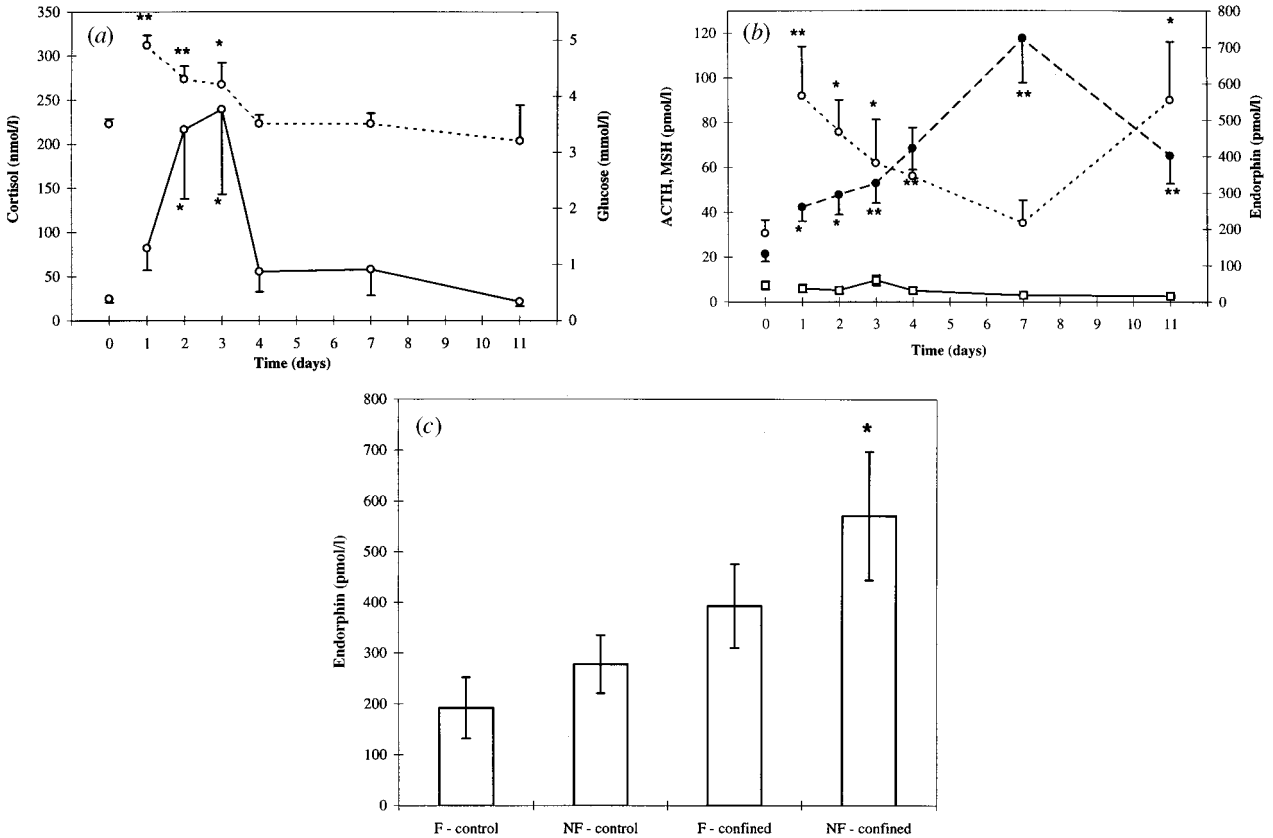

Figure 2 Changes in (a) plasma cortisol (solid line) and glucose (dotted line) concentrations and in ACTH (solid line), α-MSH (dotted line) and *N*-acetyl β-endorphin (dashed line) concentrations (*b*) of sea bream subjected to confinement for up to 11 days. Results from $t=0$ to $t=1$ day are shown in Fig. 1. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$, significant differences compared with control at $t=0$ ($n=8$; means ± S.E.M.). (c) Changes in plasma *N*-acetyl β-endorphin concentrations after 7 days fasting, confinement, or both. F-controls: fed, 4 kg/m^3; NF-controls: not fed, 4 kg/m^3; F-confined: fed, 70 kg/m^3; NF-confined: not fed, 70 kg/m^3. $^{*}P<0{\cdot}05$; significant difference compared with F-control ($n=8$; means ± S.E.M.).

cortisol to ACTH ratio is depicted in Fig. 3*c*. During confinement, plasma ACTH measurements of 1 pM were repeatedly accompanied by cortisol measurements of 5 nM, whereas, after air exposure, 1 pM plasma ACTH was accompanied by disproportionate cortisol values of up to 113 nM.

Both air exposure and confinement disturbed the hydromineral balance, although with different kinetics. Plasma osmolality had increased slightly 30 min after air exposure. A small increase in osmolality was also found in fish 8 h after the onset of the confinement (Fig. 4*a*). Thirty minutes after air exposure, plasma Na and Cl showed a similar pattern as plasma osmolality, whereas plasma Mg had doubled (Fig. 4*b*). After 1 h, these plasma mineral concentrations had returned to control values. No changes in plasma K, Ca and P concentrations were detected in air-exposed fish (results not shown). In confined fish, a delayed response (compared with air-exposed fish) was observed (Fig. 4*c*). Plasma Na and Cl concentrations had increased slightly after 8 h and remained increased up to 24 h. Plasma Mg had almost doubled after 8 h. No changes in K, Ca and P concentrations were detected at any time during short-term confinement (results not shown).

Discussion

Endocrine response

The basal plasma cortisol concentration in gilthead sea bream is comparable to that in other fish (Barton & Iwama 1991). Air exposure produced a 50-fold increase in plasma cortisol concentrations. Surprisingly, this marked increase was not accompanied by an increase in plasma ACTH or β-endorphin. This experiment was repeated three times, to exclude confounding effects of tank-related influences. Those measurements confirmed the absence of an increase in plasma ACTH concentration after air exposure. Furthermore, measurements within 30 min after air exposure did not reveal any ACTH peak, indicating that air

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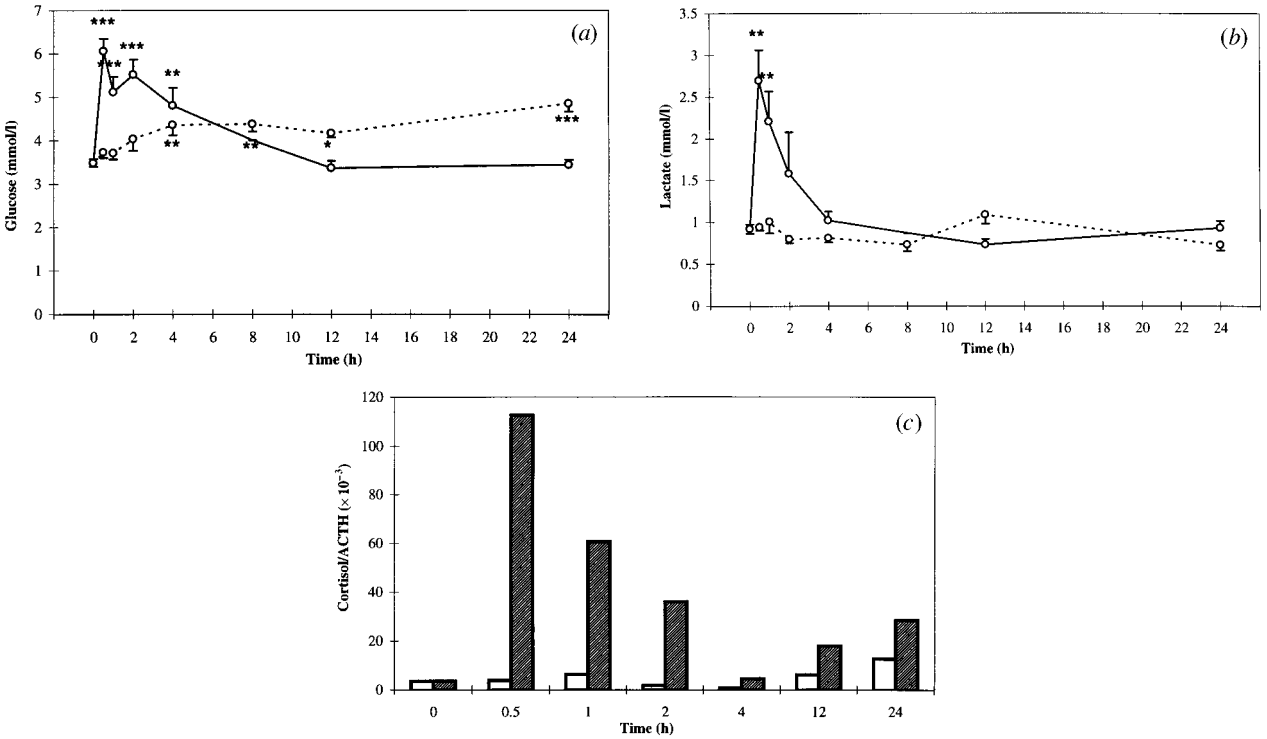

Figure 3 Changes in plasma glucose (*a*) and lactate (*b*) concentrations of sea bream subjected to 3 min air exposure (solid line), or up to 24 h confinement (dotted line). (*c*) Cortisol to ACTH ratio of sea bream subjected to 3 min air exposure (shaded bars), or up to 24 h confinement (open bars). **P*<0·05, ***P*<0·01, ****P*<0·001, significant differences compared with control at *t*=0 (*n*=8; means ± S.E.M.).

exposure induced an ACTH-independent increase in cortisol. Only an increase in α-MSH was found, indicating that air exposure activates the melanotrophic cells in the pars intermedia of the pituitary gland, but not the corticoptrophic cells in the pars distalis. Differential effects of stressors on ACTH and MSH cells have also been reported for salmonids and tilapia (Sumpter *et al.* 1985, 1986, Balm *et al.* 1994). As indicated by the increase in plasma lactate, air exposure induced acidosis by increased anaerobic muscle activity, associated with the observed avoidance behaviour. As α-MSH is released in response to a low water pH in tilapia, and as acid water causes acidosis (Lamers *et al.* 1994), we suggest that the increase in plasma α-MSH after air exposure is mediated by plasma acidosis.

What, then, triggered the increase in cortisol in air-exposed fish? The data strongly suggest that corticotrophic messengers other than ACTH are involved. As air exposure induces hypoxia, the decrease in blood oxygen content may have triggered the release of catecholamines (Reid *et al.* 1998). In eel, catecholamines released by the chromaffin cells of the head kidney stimulate, in a paracrine fashion, the interrenal cells and trigger a cortisol response (Epple *et al.* 1993). However, a direct effect of catecholamines on the cortisol release *in vivo* could not be demonstrated for carp (Gfell *et al.* 1997). Gfell *et al.* (1997) demonstrated that the head kidney of carp is influenced by the parasympathetic system acting on interrenal cells, by showing that acetylcholine stimulated cortisol secretion *in vitro*. Considering the association of interrenal and chromaffin cells in the head kidney, release of cortisol in air-exposed fish could be mediated either indirectly by chromaffin cells in a paracrine matter or directly via cholinergic activation of the interrenal cells. Thus the marked increase in plasma cortisol concentrations after air exposure may have resulted from a parasympathetic activation, rather than from POMC-derived hormones.

Confinement for up to 24 h induced an initial increase in the plasma cortisol concentration, up to eight times that of controls. Tort *et al.* (1996), demonstrated a threefold increase in plasma cortisol concentration after 2 days of confinement (5 nM in controls to 15 nM after confinement) in sea bream. The lower cortisol response in that study can be ascribed to the differences in density that the fish experienced: we increased the density to 70 kg/m^3, whereas Tort and colleagues increased it to 22 kg/m^3.

In *Sparus aurata*, the activation of the BPI axis in response to 24 h confinement appears rather transient, as plasma cortisol, ACTH and α-MSH concentrations returned to control values within a few hours. The response to 11 days confinement was particularly noticeable at days 2 and 3. The ACTH-independent increase in plasma cortisol after 2 days confinement was accompanied

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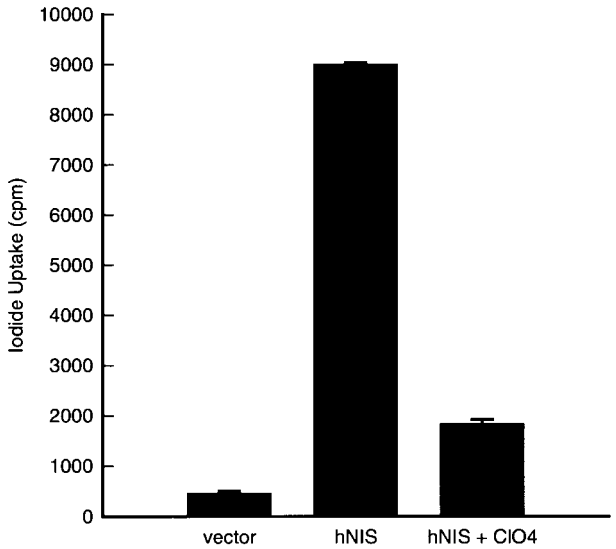

Figure 4 Effects of air exposure and confinement on plasma parameters in sea bream. (*a*) Changes in plasma osmolality in sea bream exposed to air for 3 min (solid line), or confined up to 24 h (dotted line). (*b*) Plasma Na (solid line), Cl (dotted line) and Mg (dashed line), in sea bream exposed to air for 3 min; c: plasma Na (solid line), Cl (dotted line) and Mg (dashed line) in sea bream confined for 24 h. **P*<0·05, ***P*<0·01, significant differences compared with control at *t*=0 (*n*=8; mean ± S.E.M.).

by an increase in plasma glucose concentrations, indicating that the increase in plasma cortisol had resulted from parasympathetic activation (Randall & Perry 1992). Plasma α-MSH concentrations had increased during the first days of the confinement. This increase in α-MSH by itself can not explain the ACTH-independent increase in plasma cortisol concentrations observed after 2 and 3 days confinement in *Sparus aurata*. Indeed, a similar increase in plasma α-MSH concentrations was found in the short-term (24 h) confinement experiment, in which plasma cortisol concentrations already had returned to control values. As the β-endorphin concentration was also increased in the long term, we do not exclude that synergistic effects of α-MSH and β-endorphin, as described for tilapia (Balm *et al.* 1995), triggered the release of cortisol in long-term confined gilthead sea bream.

The β-endorphin response to the 24 h confinement was less clear. We observed an increase in plasma β-endorphin coinciding with a decrease in plasma α-MSH 24 h after the onset of confinement. Assuming that, in fish (Rodrigues & Sumpter 1983) as in other vertebrates (Mains & Eipper 1979, Martens *et al.* 1980), acetylated β-endorphin is co-released with α-MSH, we speculate that, during confinement, the peripheral clearance of the peptides differs or that the acetylation machinery for β-endorphins in the MSH cells is specifically stimulated. Surprisingly, the opposite pattern was found during the second week of the experiment – that is, decreased β-endorphin concentrations and increased α-MSH-concentrations. The antibody that was used in the β-endorphin RIA specifically recognizes *N*-acetylated endorphins, which are released only by the melanotrophic cells of the pars intermedia (Takahashi *et al.* 1984). During the first 7 days of the 11-day confinement period, a strong and persistent increase in *N*-acetylated β-endorphin concentrations was demonstrated as a combined response of fasting and confinement. For mammals, it has been shown that concentrations of *N*-acetylated endorphins increase when animals are fasting. During the long-term confinement experiment, the fish were not fed and therefore the increased plasma β-endorphin concentrations can be partially related to nutritional status. Recently, an increase in acetyl-endorphin has also been demonstrated in sea bream subjected, for 1 month, to confinement and crowding (Mosconi *et al.* 1998).

We observed daily variations in plasma cortisol concentrations around feeding times. Diurnal variations in plasma cortisol concentrations and in other plasma parameters have been demonstrated in many fish species, including sea bream (Pavlidis *et al.* 1997) and variations attributable to such factors as feeding regimen and season have been reported before (Spieler & Noeske 1984, Cerda-Reverter *et al.* 1998). As daily variations at sampling times could interfere with our measurements, feeding was stopped 24 h before the start of the experiments, and this proved effective in eliminating daily variations in plasma cortisol.

Metabolic response

Clear differences were observed between the metabolic responses of the air-exposed and confinement groups. Air exposure induced a strong and rapid increase in both glucose and lacate. These results point to a sympathetic activation of the chromaffin cells in the head kidney and the release of catecholamines. Arterially infused catecholamines have been shown to induce hyperglycaemia in carp, and increased plasma lactate concentrations are associated with increased catecholamine concentrations and hypoxia (van Raaij *et al.* 1995, 1996, Fabbri *et al.* 1998). As gas exchange is compromised in air-exposed fish, the resulting hypoxia will contribute to the production of high lactate concentrations (Vijayan *et al.* 1994, Maxime *et al.* 1995). Furthermore, it has been shown for juvenile rainbow trout that 30 s handling results in an increase in plasma glucose concentrations, even in fish fed cortisol-enriched food to downregulate their cortisol-producing cells (Barton *et al.* 1987). Hepatic glycogenolysis has been shown to be a source for such a catecholamine-mediated increase in plasma glucose (Vijayan *et al.* 1994).

In confined fish, plasma glucose concentrations increased gradually and less explicitly when compared

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with the rapid and strong increase in glucose in air-exposed fish. Enhanced glycogenolysis or a decreased clearance of glucose from the blood was shown to be the source for increased plasma glucose concentrations in confined tilapia (Vijayan *et al.* 1997). Moreover, gluconeogenesis could explain the increase in plasma glucose independently of altered glucose clearance, as was reported for confined sea raven (*Hemitripterus americanus*; Vijayan & Moon 1994).

Hydromineral response

Many stressors affect the hydromineral balance in fish (Wendelaar Bonga 1997). In our experiments, a disturbance of the hydromineral balance was found in both the air-exposure and the confinement experiments, however, the magnitude and the time profile of the responses differed.

In fish stressed by air exposure, osmolality and plasma Na, Mg and Cl increased rapidly, whereas protein concentrations and plasma K, Ca and P were not affected. Apparently, only a moderate and rather specific loss of permeability control occurred during air exposure. On the basis of the changes in plasma glucose and lactate discussed above, we assume that air exposure induced a rapid catecholamine response within the 1 h time span of the experiment. Although highly increased catecholamine concentrations may have a beneficial effect by stimulating oxygen uptake via the gills, these may also result in increased permeability of the surface epithelia to water and ions (McDonald & Milligan 1992). As seawater is hyperosmotic and hyperionic to the blood plasma of *Sparus aurata*, an increased permeability of the surface epithelia to water and ions leads to an influx of ions and an efflux of water and, during severe stress, plasma proteins.

In fish stressed by confinement, plasma osmolality only tended to increase 8 h after the onset of the confinement, and small but significant increases of plasma Na, Mg and Cl occurred, whereas plasma K, Ca and P concentrations did not change notably.

Summary

We showed that air exposure and confinement have different effects on plasma cortisol, ACTH, α-MSH, β-endorphin, glucose and lactate concentrations, and on plasma Na, Cl and Mg concentrations. The observations of marked increases in plasma cortisol, glucose and lactate during air exposure indicate a stimulation of the BSC axis, with little or no activation of the ACTH and α-MSH cells. Apparently, confinement mainly stimulates the BPI axis, whereas it produces a marked increase in plasma ACTH, α-MSH and β-endorphin concentrations. Thus the stress response in sea bream is far from uniform and involves stimulus-specific pathways.

Acknowledgements

The authors gratefully acknowledge J M Naranjo, director of CICEM 'El Toruño', Junta Andalucia, El Puerto de Santa Maria 11510, Cádiz, Spain for hospitality and the generous supply of experimental fish. The authors would like to thank Dr M Valdivia for materials used in the cortisol RIA, Mr S van de Akker and Mr E van Dooren for technical assistance, and Mr F Repiso, Mr J L Morales and Mr L M Torres for technical assistance and maintenance of the experimental fish. This work was supported by the Netherlands Organization for Scientific Research (R 88–212) to R J Arends and by DGES PB96–1511 to Dr J M Mancera.

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Received 26 October 1998
Revised manuscript received 4 May 1999
Accepted 8 June 1999

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