Aquaculture Research, 2005, **36**, 172–179 doi: 10.1111/j.1365-2109.2004.01202.x

Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling

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Abstract

Understanding how gilthead sea bream, *Sparus aurata* L., an important Mediterranean Sea species for aquaculture, respond physiologically to stressors commonly encountered in intensive rearing is important for effective production, as managing for stress is a major factor in maintaining healthy fish stocks. Our objective was to determine whether holding juvenile gilthead sea bream at a high density (HD), as a chronic stressor, would affect their physiological responses to a subsequent acute handling stressor. After acclimation at a low density (LD) of 6 kg m^{-3} in 200-L circular tanks containing 33–36 g L^{-1} recirculating seawater at 19 °C under a normal photoperiod, juvenile 37-g gilthead sea bream were confined for 14 days at a HD of 26 kg m^{-3} and then subjected to 30-s aerial emersion in a dipnet. Plasma levels of cortisol, glucose, lactate, osmolality and chloride were determined in fish held in separate lots during LD (control) and HD confinement at 0, 1, 2, 7 and 14 days, and then after handling at 0, 1, 2, 4 and 8 h. Although plasma cortisol levels were similar in LD and HD fish groups after 14 d of confinement (15 and 23 ng mL^{-1}, respectively), the cortisol response in fish from the HD treatment at 1 and 2 h following acute handling (70 and 37 ng mL^{-1}, respectively) was only about half of that measured in the control group (139 and 102 ng mL^{-1}); plasma cortisol was similar in both groups by 4 and 8 h. In contrast, plasma glucose elevations in response to handling were higher at 4 and 8 h in the HD-held fish (94 and 72 mg dL^{-1}, respectively) than in those from the LD treatment (59 and 51 mg dL^{-1}); glucose responses were similar in both groups at 1 and 2 h after handling and throughout confinement. Plasma lactate levels were higher in LD fish than in the HD group at the beginning of the experiment but were similar after 14 d confinement and responses to handling were similar (e.g. 33 and 35 mg dL^{-1} at 1 h). Plasma osmolality showed increases during the first 2 h after acute handling but no differences were evident between the two density treatments at any time during confinement or posthandling. Plasma chloride levels did not change throughout the experiment. The reduced plasma cortisol response to acute handling likely resulted from negative feedback of mildly but chronically elevated circulating cortisol caused by the confinement stressor on the hypothalamic–pituitary–interrenal axis. While other post-handling physiological changes also showed differences between treatment groups, the suppressed cortisol response in the HD-held fish suggests a reduction in the gilthead sea bream's normal capacity to respond to an acute stressor.

Keywords: stress, handling, confinement, cortisol, glucose, lactate, sea bream, *Sparus aurata*

Introduction

Characteristic endocrine and other physiological responses of teleostean fishes to stressors typically encountered in aquaculture have been studied thoroughly during the last two decades (Barton & Iwama 1991; Iwama, Pickering, Sumpter & Schreck 1997; Wendelaar Bonga 1997). Such responses

include elevations in plasma concentrations of cortisol, glucose, lactate and, in marine species, osmolality and major ions, and have become well established as useful indicators of the degree of acute stress experienced by fish (Barton, Morgan & Vijayan 2002). These stress responses, particularly changes in circulating cortisol, however, can be modified extensively by genetic, developmental and internal and external environmental factors (Barton 2002). Prior exposure to other stressors can appreciably alter the response of cortisol to acute stress, for example. The presence of pollutants or contaminants in the water sufficient to chronically elevate plasma levels of cortisol in fish has been shown to attenuate the response to an acute stressor (Hontela, Rasmussen, Audet & Chevalier 1992; Norris, Donahue, Dores, Lee, Laldonado, Ruth & Woodling 1999; Laflamme, Couillard, Campbell & Hontela 2000). This effect possibly occurs as a result of either down-regulation of the hypothalamic–pituitary–interrenal (HPI) axis from continuous negative feedback of cortisol or direct toxic action of the chemical stressor on the axis' functional integrity (Hontela 1997). A reduced corticosteroid response following an acute stressor has been mimicked by prior continuous treatment with cortisol-impregnated feed, demonstrating the negative-feedback effect of elevated circulating cortisol on the HPI axis (Barton, Schreck & Barton 1987; Rotllant, Arends, Mancera, Flik, Wendelaar Bonga & Tort 2000) or on related cellular mechanisms (Basu, Kennedy, Hodson & Iwama 2002).

An altered interrenal response to an acute stressor resulting from chronic stress has been shown in fish exposed to chemical stressors but few studies have demonstrated this phenomenon in fish subjected to a chronic physical stressor such as the continuous crowding or confinement experienced in intensive aquaculture. Pickering and Pottinger (1987) found that continuous crowding suppressed the subsequent cortisol response to an acute stressor in brown trout, *Salmo trutta* L., but suggested that this effect was caused by water quality changes and not the crowding *per se*. Recently, Haukenes and Barton (2004) showed that confining yellow perch, *Perca flavescens* (Mitchill), reduced their cortisol response to an acute lipopolysaccharide (LPS) challenge. In this study, we compared the physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., with an acute handling stressor after holding them at low and high tank densities. Previous studies have documented various responses of this species to handling and confinement stressors (Rotllant, Balm, Pérez-Sanchez, Wendelaar Bonga & Tort 2001; Tort, Montero, Robaina, Fernández-Palacios & Izquierdo 2001; Rotllant, Montero, Cabellero, Robaina, Izquierdo & Tort 2003). Our objective was to determine whether gilthead sea bream subjected to a high-density (HD) confinement stressor would show reduced responses, particularly in cortisol, to a subsequent acute stressor compared with those in fish held at a low density (LD). Further understanding of the nature of this species' responses to aquaculture-related stressors is important practically as gilthead sea bream is one of the most commonly used fishes for commercial marine aquaculture in the Mediterranean Sea (Gasca-Leyva, León & Hernández 2003).

Materials and methods

Acclimation and experimental conditions

Approximately 260 juvenile gilthead sea bream were obtained from Aquadelt Fish Farms, Sant Carles de la Ràpita, Spain. The fish were transported to aquarium facilities at the Universitat Autònoma de Barcelona where they were divided equally into eight rectangular fibreglass tanks. About 33 fish per tank were held in 200 L of 33–36 g L^{-1} seawater at 19 °C and a density of 6 kg m^{-3} for 2 weeks of acclimation before the experiments. Water was recirculated by a small submersible aquarium pump (Enheim, Berlin, Germany) in each tank that passed the water through an individual biofilter unit containing nitrifying bacteria to remove ammonia wastes. During acclimation and experiments, un-ionized ammonia levels [uNH_3-N, determined from dissociation tables developed by Colt (2001) from measured total ammonia-nitrogen (TAN) and compensated for temperature, pH and salinity] ranged from <0.001 to 0.024 mg L^{-1}; pH ranged from 6.35 to 8.13; and nitrite (NO_2-N) levels ranged from 0.08 to 2.85 mg L^{-1} among all tanks (Table 1). Water was vigorously aerated continuously with compressed air supplied by a common diaphragm pump and delivered through two airstones in each tank. Tanks were kept partially covered under a controlled photoperiod of 12 h light:12 h dark using artificial light, with the light period commencing at 07:00 hours. The fish were fed a maintenance ration of about 0.3–0.5% body weight per day with an experimental sea bream growth diet (INVE Technologies NV, Baasrode, Belgium); feeding ceased 24 h before fish were sampled from designated tanks. Any mortality during acclimation and experiments was recorded and those fish were removed from the tanks.

Table 1 Salinity range, and range (low–high) and mean (± SE, $n = 9$) of measured pH, nitrite-nitrogen (NO_2-N) and total ammonia-nitrogen (TAN), and calculated un-ionized ammonia-nitrogen (uNH_3-N) in tanks containing gilthead sea bream at low (LD) and high (HD) densities throughout the experiments

Tank	Salinity (g L^{-1})	pH	NO_2-N (mg L^{-1})	TAN (mg L^{-1})	uNH_3-N (mg L^{-1})
LD 1	33–35	7.50–7.96	0.31–2.16	0.03–0.23	<0.001–<0.008
		7.72 ± 0.06 a,b	0.80 ± 0.22 a,b	0.10 ± 0.02 a	0.002 ± <0.001 a,b
LD 2	34	6.70–7.80	0.11–0.43	0.03–0.12	<0.001–<0.001
		7.48 ± 0.10 a,b	0.19 ± 0.03 a	0.07 ± 0.01 a	<0.001 ± <0.001 a
LD 3	36–37	7.10–7.90	0.12–0.34	0.01–0.14	<0.001–<0.002
		7.50 ± 0.08 a,b	0.23 ± 0.03 a	0.07 ± 0.01 a	<0.001 ± <0.001 a
LD 4	33–34	7.47–7.80	0.08–2.70	0.03–2.61	<0.001–<0.022
		7.69 ± 0.04 a,b	1.54 ± 0.29 b	0.80 ± 0.33 b,c	0.007 ± 0.003 b,c
HD 1	34–36	6.35–7.93	0.51–2.72	0.03–2.44	<0.001–<0.024
		7.39 ± 0.18 a	1.53 ± 0.77 b	1.16 ± 0.32 c	0.011 ± 0.003 c
HD 2	34–36	6.80–7.86	0.14–2.85	0.03–1.79	<0.001–<0.016
		7.60 ± 0.11 a,b	1.11 ± 0.38 a,b	0.38 ± 0.21 a,b	0.004 ± 0.002 a,b
HD 3	34–36	7.60–7.92	0.50–1.39	0.07–0.09	<0.001–<0.004
		7.73 ± 0.04 b	0.83 ± 0.10 a,b	0.20 ± 0.04 a,b	0.002 ± <0.001 a,b
HD 4	35	7.61–8.13	0.24–2.77	0.01–0.84	<0.001–<0.007
		7.78 ± 0.05 b	1.55 ± 0.36 a,b	0.23 ± 0.10 a,b	0.003 ± 0.001 a,b

Mean values followed by letters not in common indicate significant difference ($P < 0.05$) within that parameter column.

Chronic confinement stressor

For the chronic HD confinement treatment, four tanks containing 33 juvenile sea bream per tank (mean weight 37.1 g) were held at a density of 26 kg m^{-3} for 14 days. Water was removed to alter densities initially, and subsequently after fish removal to maintain densities constant, by using a siphon, which allowed for minimal disturbance of the fish in the tanks. The remaining four tanks containing 33 fish per tank at an acclimation density of 6 kg m^{-3} served as LD control groups.

Five fish were removed carefully to avoid unduly disturbing the remaining fish from each of two of the four tanks ($n = 10$) for both LD and HD treatments at the onset of the experiment (day 0). Five fish per tank were then removed from the same two tanks on day 2 only and from the other two tanks on days 1 and 7 only for each treatment. This approach was adopted to keep any possible acute disturbance to remaining fish resulting from fish removal at a minimum; all groups, thus, had at least 2 days to recover physiologically from any such disturbance and both density groups were treated in the same manner. Similar sampling techniques have been used successfully with salmonid fishes with no measurable effect on physiological constituents occurring in remaining unhandled control fish (Barton, Peter & Paulencu 1980; Barton, Weiner & Schreck 1985; Barton, Schreck & Sigismondi 1986). On day 14, two to three fish per tank were taken from all four tanks ($n = 10$) for each treatment just before commencing the acute handling experiment; these samples also served as hour 0 samples for acute handling.

Acute handling stressor

After 14 days of HD or LD confinement, fish in all tanks were subjected to an acute handling stressor by holding them in the air in a net for 30 s. Fish from each treatment group were then divided equally into eight tanks per treatment in order to provide separate replicate sample groups at post-handling times without disturbing remaining fish. Water volumes in tanks were kept similar to those used for the LD and HD chronic treatments accordingly. Five fish from each of two tanks ($n = 10$) were sampled at 1, 2, 4 and 8 h after handling.

Fish sampling

All fish were sampled by first placing them in a lethal concentration of 2-phenoxy-ethanol (Sigma, St Louis, MO, USA) after being removed from the tank with a hand-net; fish were immobilized in < 1 min. Blood was obtained from the caudal vasculature using a 1-mL syringe equipped with a 25-G needle (Houston 1990) and transferred to a 1.5-mL microcentrifuge tube containing 5% sodium heparin. All sampling was completed within 5 min of fish removal from

the tank. Plasma was separated by 5 min centrifugation and stored at − 25 °C for subsequent analysis of cortisol, glucose, lactate, osmolality and chloride.

Sample and data analysis

Plasma cortisol levels were measured by radioimmunoassay following the procedure described for gilthead sea bream in Rotllant and colleagues (2001) using antibody purchased from BioLink SL (Barcelona, Spain). Plasma glucose and lactate were determined by enzymatic colorimetric methods in ELISA plates using commercial kits (BioMérieux 61270 and 61192 for glucose and lactate, respectively; BioMérieux SA, Marcy-l'Etoile, France). Plasma osmolality was assessed by direct reading using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). Plasma chloride was measured using a Corning model 925 chloridometer (CIBA-Corning, Medfield, MA, USA).

Two-way analyses of variance (ANOVA) were conducted with ProStat (Poly Software International, Salt Lake City, UT, USA) using tank means as independent observations to compare treatments and times for the chronic confinement period and the acute handling trial separately. Two-way ANOVA were carried out of water quality parameters to detect differences among tanks and through time. *Post hoc* comparisons to compare means were made using Duncan's Multiple-Range Test with significance level at $P = 0.05$.

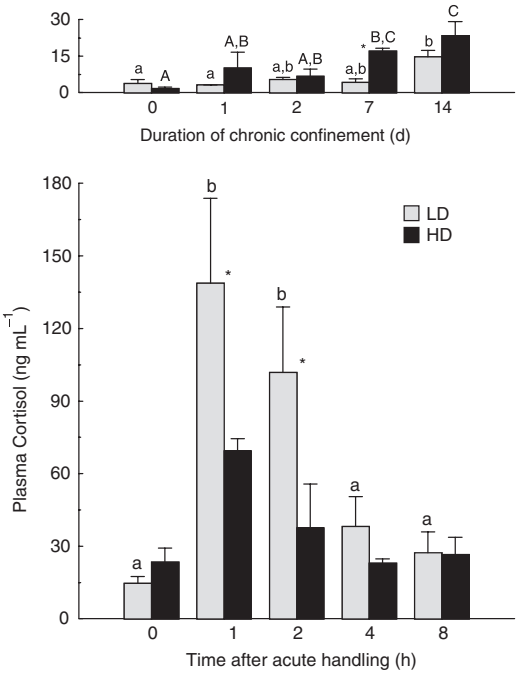

Figure 1 Mean (+SE) levels of plasma cortisol (ng mL^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. Value bars for 14-day confinement are also those for 0 h handling. Bars accompanied by letters not in common indicate significant difference ($P < 0.05$) from other values at different times for that treatment; lower case letters are used for LD treatments and capital letters for HD treatments for ease of interpretation. An asterisk (*) accompanying a pair of bars indicates a significant difference between the two treatments at that time. Bars within a treatment series not accompanied by letters indicate no significant difference among all times.

Results

Chronic confinement

Plasma cortisol increased in both LD- and HD-treatment groups during the 14-day confinement period (Fig. 1); an elevated level of plasma cortisol was apparent by day 7 in the HD fish but not until day 14 in those held at LD. A significant difference in plasma cortisol between LD and HD fish occurred only on day 7 (Fig. 1).

Concentrations of plasma glucose were similar in both sea bream groups throughout the confinement period with no significant differences evident among times within either density treatment or between treatments (Fig. 2). Plasma lactate levels were more variable than glucose; levels were generally lower in the HD group than in the LD fish during confinement and significantly so on day 0 (start of experiment) (Fig. 3). Whereas plasma lactate remained constant in the HD fish, it was significantly lower at day 14 than at day 0 in the LD-treatment group (Fig. 3).

Sea bream in both LD and HD treatments exhibited a significant reduction in plasma osmolality by day 2 compared with that at the start of the experiment but returned to initial (day 0) levels by day 14 (Fig. 4). Plasma chloride concentrations ranged from 154 ± 4.0 (± SE) to 163 ± 0.2 mEq L^{-1} in the LD group and from 158 ± 1.3 to 160 ± 0.3 mEq L^{-1} in the HD group during the 14-day confinement period; no significant differences were evident among times or between treatments (data not shown).

No mortality was observed during the acclimation period but three HD tanks had fish mortalities during

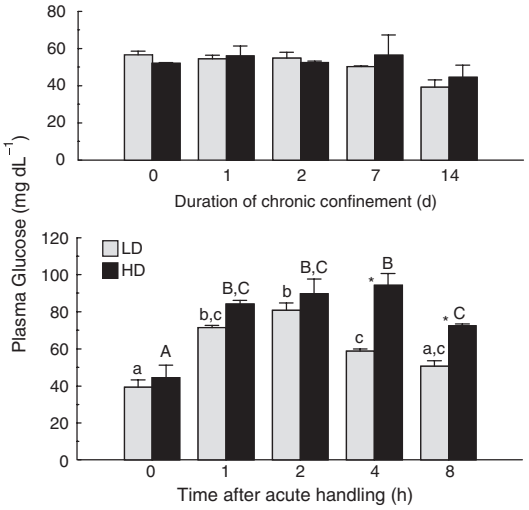

Figure 2 Mean (+SE) levels of plasma glucose (mg dL^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. See Fig. 1 for explanation of letter symbols accompanying the value bars. Bars within a treatment series not accompanied by letters indicate no significant difference among all times.

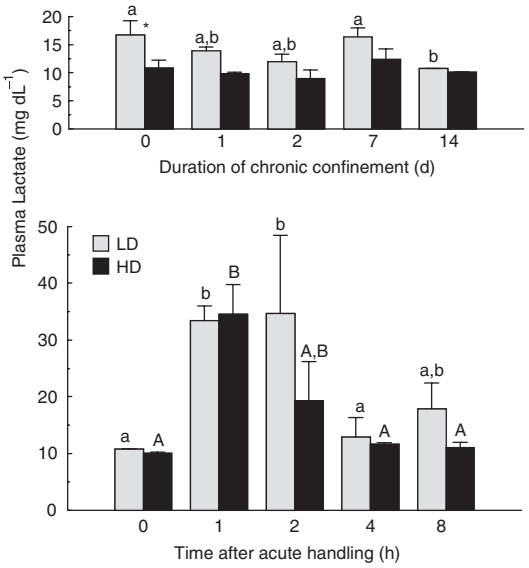

Figure 3 Mean (+SE) levels of plasma lactate (mg dL^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. See Fig. 1 for explanation of letter symbols accompanying the value bars. Bars within a treatment series not accompanied by letters indicate no significant difference among all times.

chronic confinement [HD1 = 2 (6.1%), HD2 = 6 (18%), HD3 = 1 (3.0%)]. No fish mortality occurred in LD tanks.

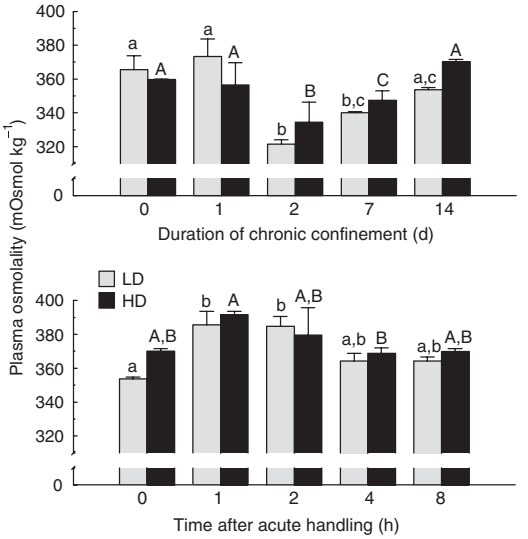

Figure 4 Mean (+SE) levels of plasma osmolality (mOsmol kg^{-1}) in juvenile gilthead sea bream during 14 d of confinement at low (LD) or high (HD) density followed by a 30-s acute handling stressor. See Fig. 1 for explanation of letter symbols accompanying the value bars.

Acute handling

Gilthead sea bream from the LD treatment had significantly elevated levels of plasma cortisol after being subjected to 30 s of handling; these changes were evident at 1 and 2 h after handling (Fig. 1). Plasma cortisol concentrations in fish from the HD treatment, although apparently elevated at 1 h, were not significantly different from each other throughout the post-handling period (Fig. 1).

Plasma glucose in sea bream from both treatments increased in a similar fashion during the first 2 h after handling (Fig. 2). At 4 and 8 h post handling, plasma glucose levels remained significantly higher in the HD fish than in the LD group (Fig. 2). Plasma lactate also increased significantly by 1 h after handling in both groups and concentrations remained elevated in the LD group at 2 h compared with those in the HD fish (Fig. 3). Plasma lactate levels in both groups at 4 and 8 h were similar to those before handling (0 h).

Plasma osmolality increased significantly in the LD fish during the first 2 h after fish were handled, but returned to pre-handling levels by 4 h (Fig. 4). Post-handling changes in plasma osmolality in the HD fish were not significant except at 1 h compared with that at 4 h (Fig. 4). Treatment groups were similar at all post-handling times. Concentrations of plasma chloride ranged from 157 ± 0.6 to

169 ± 1.9 mEq L^{-1} in sea bream held at LD and from 159 ± 0.8 to 168 ± 1.4 mEq L^{-1} in the HD group from the time of initial handling up to 8 h, the end of the trial; no significant differences occurred among times or between treatments (data not shown).

No fish mortality occurred in HD or LD tanks during the acute handling experiment.

Discussion

Pre-stress and post-acute stress responses of plasma cortisol, glucose, lactate and osmolality in the gilthead sea bream used in this study were similar in magnitude to those previously shown for this species (Rotllant *et al.* 2001; Tort *et al.* 2001). Plasma chloride remained unchanged and levels were typical of those in other marine teleosts, as was osmolality (Cech Jr 2000). An explanation for the decline in plasma osmolality after 2–day confinement in both groups of fish is not forthcoming as salinity levels remained relatively constant. We noticed, however, that fish did not start actively taking feed until a few days after their initial transfer and, as suggested by Cech Jr (2000), the short-term dietary deprivation could have altered blood ion levels. Nevertheless, plasma osmolality levels in both groups were similar to their pre-transfer values by the beginning of the handling experiment on day 14.

Elevations in plasma cortisol in the sea bream held under HD confinement for 2 weeks and then handled for 30 s were clearly reduced (e.g. at 1 and 2 h) compared with those in the control group. This phenomenon has been demonstrated very little in fish subjected to a chronic physical stressor, such as confinement, before being acutely handled. In one other study, Haukenes and Barton (2004) showed a trend through time towards reduced corticosteroid responses in yellow perch subjected to LPS treatment that were first either confined at HD or confined and then allowed a day to recover at LD compared with those that were held at the ambient LD. In a similar aquaculture setting, Pickering and Pottinger (1987) reported a reduced elevation of plasma cortisol in handled and acutely confined brown trout after being held in a crowded condition, but indicated that this was likely a result of being exposed chronically to high concentrations of un-ionized ammonia in the tanks. In that case, those authors concluded that continuous interrenal activity caused a down-regulation of the HPI axis from negative feedback by circulating cortisol, which attenuated the response to an additional stressor. In this experiment, and that by Haukenes and Barton (2004), we suspect that a similar phenomenon is occurring as a result of holding the fish at a HD.

Alternatively, the fish may have become sufficiently desensitized to the chronic stressor such that their overall response to the additional stressor was suppressed, similar to what Barton and colleagues (1987) found in rainbow trout, *Oncorhynchus mykiss* (Walbaum), subjected to mild acute stressors daily for a number of weeks. Both plasma cortisol and glucose elevations in response to handling at the end of the 10-week treatment period in that study were lower compared with those in control groups, suggesting not only desensitization of the HPI axis but of the adrenergic mechanisms controlling stress-induced hyperglycaemia as well (Reid, Bernier & Perry 1998). In the present investigation, plasma glucose increased by comparable amounts following 30 s of handling in both LD- and HD-held gilthead sea bream, however, and elevated concentrations were sustained at 4 and 8 h post handling in the HD group, which suggests that stress-response mechanisms were not 'desensitized' in that context. The similarity of increases in plasma lactate and osmolality levels in LD and HD fish at 1 h further support our view that both groups experienced a similar degree of acute stress from being handled. The fish from the HD treatment may have already experienced a higher degree of stress during the 14-day confinement period before handling than those in the LD group as suggested by higher plasma cortisol levels at day 7 and by the fact that three HD tanks had fish mortality whereas the LD tanks had none. Extended periods of confinement have been shown to induce elevations of circulating cortisol in salmonid fishes similar to those we measured in the sea bream (Pickering & Stewart 1984; Pickering, Pottinger, Carragher & Sumpter 1987; Pickering & Pottinger 1989).

The suppression of the acute corticosteroid response in fish following their continued exposure to water-borne contaminants is a well-documented phenomenon (Hontela *et al.* 1992; Brodeur, Daniel, Ricard & Hontela 1998; Wilson, Vijayan, Kennedy, Iwama & Moon 1998; Norris *et al.* 1999; Laflamme *et al.* 2000). Hontela (1997) considered that this interrenal impairment could be due to either down-regulation of the HPI axis by continual negative feedback, as discussed, or as a result of direct toxic action of the compound on the cellular mechanisms or function of the interrenal tissue itself, depending on the type of contaminant. As sea bream were not exposed to a

chemical stressor *per se* in this study, the latter explanation for the attenuated cortisol response in the HD fish is unlikely. It is possible, however, that changes in water chemistry as a result of confinement may have affected interrenal responsiveness by acting as an additional stressor along with HD confinement. Pickering and Pottinger (1987) concluded that confinement-induced alterations in dissolved oxygen (O_2), free carbon dioxide (CO_2) and TAN may have acted in combination to cause a reduction in the response of plasma cortisol to an acute 1-h confinement stressor. In our experiments, the confinement period was for 14 days, thereby allowing for acclimation to occur, and Pickering and Stewart (1984) showed previously that mild but chronic elevation of plasma cortisol from HD confinement can occur independent of water chemistry changes. We concluded that this was likely the case in our study as the water quality parameters of pH, NO_2-N, TAN and uNH_3-N, while showing some variation throughout the experiment, were not appreciably different among the tanks or between the LD and HD groups overall; free CO_2 and dissolved O_2 were not measured, but tanks were vigorously aerated continuously. Moreover, levels of nitrogenous parameters remained well below lethal levels for fish (Tomasso 1994) and those shown to chronically elevate plasma cortisol (Sykes 1999).

Despite considerable research in this area, the implication for a reduced endocrine stress response in fishes used for aquaculture is still not clear. While it may be advantageous to select fish exhibiting low stress responses for intensive commercial aquaculture (Fevolden, Reftsie & Røed 1991; Pottinger, Moran & Morgan 1994), those being reared for stock enhancement could conceivably be at a disadvantage when released into a natural environment because of a lowered capacity to mount the appropriate response to cope with additional stressors. For cage-culture of gilthead sea bream, a possible impairment of the corticosteroid stress response from rearing at overly high densities could result in a reduction in their physiological ability to cope with social stressors from conspecifics or abiotic changes in their confined environment.

Acknowledgments

The authors sincerely thank Isasi Gairin and staff at Aquadelt Fish Farms, Sant Carles de la Ràpita (Tarragona), for generously donating the juvenile gilthead sea bream for the study. The authors are also grateful for the technical assistance of Judit Virtudes, Universitat Autònoma de Barcelona, for water quality monitoring during the experiments and Diedra Clausen, University of South Dakota, for plasma chloride analyses. B.A.B. was supported by a Visiting Professor grant from the Generalitat de Catalunya through the Universitat Autònoma de Barcelona while on a sabbatical leave from the University of South Dakota.

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