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Hypo-osmotic stress-induced physiological and ion-osmoregulatory responses in European sea bass (*Dicentrarchus labrax*) are modulated differentially by nutritional status

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ABSTRACT

We investigated the impact of nutritional status on the physiological, metabolic and ion-osmoregulatory performance of European sea bass (*Dicentrarchus labrax*) when acclimated to seawater (32 ppt), brackish water (20 and 10 ppt) and hyposaline water (2.5 ppt) for 2 weeks. Following acclimation to different salinities, fish were either fed or fasted (unfed for 14 days). Plasma osmolality, [Na⁺], [Cl⁻] and muscle water content were severely altered in fasted fish acclimated to 10 and 2.5 ppt in comparison to normal seawater-acclimated fish, suggesting ion regulation and acid–base balance disturbance. In contrast to feed-deprived fish, fed fish were able to avoid osmotic perturbation more effectively. This was accompanied by an increase in Na⁺/K⁺-ATPase expression and activity, transitory activation of H⁺-ATPase (only at 2.5 ppt) and down-regulation of Na⁺/K⁺/2Cl⁻ gene expression. Ammonia excretion rate was inhibited to a larger extent in fasted fish acclimated to low salinities while fed fish were able to excrete much more efficiently. Consequently, the build-up of ammonia in the plasma of fed fish was relatively lower. Energy stores, especially glycogen and lipid, dropped in the fasted fish at low salinities and progression towards the anaerobic metabolic pathway became evident by an increase in plasma lactate level. Overall, the results indicate no osmotic stress in both feeding treatments within the salinity range of 32 to 20 ppt. However, at lower salinities (10–2.5 ppt) feed deprivation tends to reduce physiological, metabolic, ion-osmoregulatory and molecular compensatory mechanisms and thus limits the fish's abilities to adapt to a hypo-osmotic environment.

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1. Introduction

Salinity is a limiting factor for many marine organisms. Owing to global warming, glaciers and ice caps have been rapidly disappearing, and more frequent intense rainfall events occur. Consequently, the salinity gradient of some marine ecosystems such as enclosed bays, estuaries and the inshore waters has gradually reduced over the last few decades (Wong et al., 1999; Freeland and Whitney, 2000; Pierce et al., 2012). Moreover, euryhaline teleosts, including diadromous and non-diadromous fish, often encounter osmotic challenges at different stages of their life cycle. The shift in the ambient salinity can alter overall fitness of fish with a severe challenge to ion-osmoregulation (Kelly and Woo, 1999; Roessig et al., 2004; Yan et al., 2004). A high plasticity of the ion-transporting ability is thus necessary to cope with a wide range of environmental salinities. Ion and acid–base balance in fish is mainly mediated by the gills, kidneys and intestines. Among them, the gills, which are exposed to external environments, are the major organ for balancing ion movement between the internal milieu and the external environment. Mitochondrion-rich cells (MRCs or chloride cells) present

in the gill epithelium are the main site for active ion uptake and secretion, and these MRCs can modulate ion transport mechanisms to ensure intracellular homeostasis during osmotic challenges. When transferring marine teleosts to salinities below the iso-osmotic point, Na⁺ and Cl⁻ transport across the gill epithelia switches from excretion to uptake (Marshall and Grosell, 2005). These ion transport mechanisms in marine teleosts are coordinated by ion channels, cotransporters (e.g., Na⁺/K⁺/2Cl⁻, Na⁺/Cl⁻) and energy dependent ion transport enzymes like the Na⁺/K⁺-ATPase (NKA) (Hwang et al., 2011; Hiroi and McCormick, 2012). NKA is present on the basolateral membrane of branchial epithelium that actively transports Na⁺ out and K⁺ into the animal cells. It plays a key role in maintaining intracellular homeostasis by providing a driving force for many ion-transporting systems in the gills (Marshall and Bryson, 1998; Hirose et al., 2003; Lin et al., 2004a,b; Hwang and Lee, 2007). In teleosts and in diadromous species, NKA activity, abundance and its expression are often suggested as indicators of the osmoregulatory ability (Jensen et al., 1998; Marshall, 2002; Evans et al., 2005; Giffard-Mena et al., 2008). NKA generates a low intracellular Na⁺ gradient; thereby inducing the transport of Na⁺, K⁺ and Cl⁻ into the cell through the presence of a basolateral Na⁺/K⁺/2Cl⁻ cotransporter (NKCC), apical Na⁺/Cl⁻ cotransporter (NCC) (Hiroi et al., 2008; Hsu et al., 2014) and an apically located Cl⁻ channel

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homologous to the human cystic fibrosis transmembrane conductance regulator (CFTR) (Hwang et al., 2011; Hiroi and McCormick, 2012). NKCC seems to play a central role in the maintenance of the electrolyte content, transepithelial ion and water movement in polarised cells (Russell, 2000; Cutler and Cramb, 2001). As such, its pattern of expression at different osmoregulatory sites dictates the adaptation of teleosts to osmotic challenge (Lorin-Nebel et al., 2006).

The capacity of euryhaline fish to adapt to varying environmental salinities depends on the activation of ion transport process. The synthesis and operation of ion transport proteins impose energetic regulatory costs for active uptake or secretion of ions. Therefore, a sufficient and timely energy supply is a prerequisite for the operation of ion/osmoregulatory mechanisms in fish gills to cope with a wide range of environmental salinities through a series of coordinated physiological processes that facilitate switching between hyperosmotic and hyposmotic environment (or vice versa) (Boeuf and Payan, 2001; Chang et al., 2007; Tseng and Hwang, 2008; Evans, 2010). The energy requirement of the branchial cell is reported to be maintained by oxidation of glucose obtained from the circulation (Mommensen et al., 1985). In this context, the quantification of energy stores (e.g., glycogen, lipid and protein) in tissues would offer a better understanding of metabolic fuel supply to the ion-osmoregulatory system. Consequently, nutritional status of fish can have a pronounced impact on performance, and determine the competency to adapt to changing environments.

Periods of nutrient deprivation is a natural phenomenon in wild populations of fish especially during reproduction and migration and also occurs regularly for the cultured fish. It has already been demonstrated that energy deficiency due to feed deprivation can adversely affect numerous physiological systems such as energy supply, metabolism, ionic balance and endocrine functions (Gaylord et al., 2001; Small et al., 2002; Peterson and Small, 2004; Small, 2005; Small and Peterson, 2005; Bucking and Wood, 2006a,b). The majority of the research has investigated the impact of salinity stress alone on the adaptive responses in fish (Jensen et al., 1998; Lin et al., 2003; Lorin-Nebel et al., 2006; Giffard-Mena et al., 2008); the assessment of such responses when fish are subjected to additional ecologically relevant stressful conditions is rather scarce. However, some work has been done looking at the interaction of starvation with the salinity changes in marine fish but they were mainly focused on survival (Woo and Murat, 1981; Rodríguez et al., 2005). Therefore, the present experiment was designed to study the metabolic, physiological and ion-osmoregulatory responses in fish, which occur as compensatory mechanisms to deal with salinity variations in combination with feed deprivation.

The fish gill is also the site of excretion of excess nitrogen in the form of ammonia, and models suggest that ion regulation is also linked to the ammonia excretion pathways (Wilkie, 1997, 2002; Wright and Wood, 2009). Basolateral transporters such as NKA and NKCC are primarily associated with ion transport, but their importance in ammonia excretion has also been implicated since similarities in the hydration radius of K^+ and NH_4^+ allow substitution at transport sites (Wilkie, 1997; Randall et al., 1999; Alam and Frankel, 2006). Although the bulk of ammonia transport is thought to be through NH_3 diffusion either directly or through Rhesus proteins, intracellular NH_4^+ can be extruded across the apical membrane, presumably via a Na^+/H^+ antiporter, with NH_4^+ substituting for the H^+ (Wilkie, 1997). In this scenario, it can be presumed that an alteration in ion- and osmoregulation during individual and/or combined effect of salinity stress and starvation may also hamper internal ammonia homeostasis.

The European sea bass (*Dicentrarchus labrax* L.) is a marine teleost, is widely distributed throughout the Europe and extensively used for aquaculture and is therefore of great commercial and ecological importance. It is euryhaline at all developmental stages and in the wild, they move seasonally between the open sea and hyposaline environment such as lagoons/estuaries. This requires a modification in the ion-osmoregulatory response (Barnabé et al., 1976; Kelley, 1988). Therefore, in the present study, we used juveniles of European sea

bass as a test organism to examine how this species manipulates its various biological processes in order to retain body homeostasis when confronted with different stressors such as salinity and feed deprivation at the same time. Overall, we hypothesised that sea bass would be more affected in hypo-osmotic environments, that feeding would improve the capacity of sea bass to retain their ionic balance and ammonia homeostasis and that it would provide the necessary energy for different compensatory responses, thus facilitating acclimation to lower salinities.

To achieve our goals, we assessed (i) osmoregulatory ability by measuring ion concentration in plasma, activity and expression of branchial NKA, NKCC gene expression, H^+ -ATPase activity and muscle water content; (ii) energy budgets—glycogen, lipid and protein reserves were quantified as indicators of energy use; (iii) ammonia and urea homeostasis, by determining ammonia and urea accumulation in plasma and their excretion rate, as an indicator of nitrogen metabolism and excretion; and (iv) plasma lactate as an indicator of anaerobic metabolism.

2. Materials and methods

2.1. Experimental system and animals

European sea bass (*Dicentrarchus labrax*) juveniles (14–18 g) were obtained from Ecloserie Marine (Gravelines, France). Fish were kept at the University of Antwerp in tanks (1000 L), filled with artificial seawater (Meersalz Professional Salt, 32 ppt salt) for at least a month. Thereafter, a total of 240 fish were distributed into eight 200 L tanks ($n = 30$ per tank; 32 ppt) equipped with a recirculating water supply in a climate chamber where temperature was adjusted at $17 \pm 1^\circ C$, and photoperiod was set at 12 h light and 12 h dark. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. Fish were acclimated to the above-mentioned constant salinity, temperature and photoperiod for 3 weeks prior to the experiment and were fed with commercial pellets (Skretting, Boxmeer, The Netherlands) at a rate of 2% of their wet body weight/day.

After 3 weeks, fish were progressively exposed to three experimental salinities: 20 ppt (~500 mOsm/kg, pH 8.17; 2 tanks), 10 ppt (~249 mOsm/kg, pH 8.10; 2 tanks) and 2.5 ppt (~69 mOsm/Kg, pH 7.87; 2 tanks). Changes in salinity were progressed by reducing the salinity by 5% each 3 days until the desired salinity was reached. Fish maintained at normal seawater salinity 32 ppt (~800 mOsm/Kg, pH 8.25; 2 tanks) were designated as control group. Each experimental group and the control were acclimated at the desired salinity for 2 weeks and were fed daily at a rate of 2% on their wet body weight. Experimental salinities were adjusted by diluting artificial seawater with filtered freshwater, and salinity was measured using a hand-held refractometer.

After being acclimated at the respective salinities for 2 weeks, feeding was withheld for 14 days in one of the respective tank for each of the salinity group while feeding (2% bodyweight/day) was continued in the respective parallel tank. Water was exchanged by 40–60% twice a week to remove uneaten food and minimise the build-up of nitrates and nitrites. The salinity was tested and controlled daily by adding clean water (of the appropriate salinity) to compensate for the loss of water by evaporation. All animal experiments were approved by the local ethics committee, University of Antwerp, and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations.

2.2. Experimental protocol and excretion measurement

After 14 days of feeding or fasting, 16 fish (8 fed and 8 fasted) were transferred from their respective salinity-acclimated tank to individual 8 L glass aquaria (water volume set to 5 L) with the salinity matching 206

the acclimation salinity. The experimental aquaria were shielded with black plastic to minimise visual disturbance and fitted with individual air-stones. These aquaria were placed in a climate chamber having the same temperature and photoperiod as for the fish holding. Fish were placed in glass aquaria the evening before the sampling and left overnight to settle with continuous aeration. For the ammonia and urea excretion measurements, initial water samples (duplicate 2 mL) were taken the subsequent morning followed by a final water sample collection after 12 h. During the excretion measurements, fish were not fed.

Ammonia excretion rates J_{Amm} ($\mu\text{mol/g/h}$) were calculated as follows:

$$J_{\text{Amm}} = ([\text{Amm}]_i - [\text{Amm}]_f) \times V / (t \times M)$$

where $[\text{Amm}]_i$ and $[\text{Amm}]_f$ are the initial and final concentrations of ammonia in the water (in $\mu\text{mol/L}$) obtained from comparison to a standard curve. V indicates volume (L), t time (h) and M mass (g). Urea excretion rates (J_{urea}) were calculated as for J_{Amm} .

2.3. Sampling procedure

Following the last water sampling, the animals were terminally anaesthetised with a lethal dose of neutralised (with 2 parts NaOH) MS-222 (ethyl 3-aminobenzoate methane-sulphonic acid, 1 g/L, Acros Organics, Geel, Belgium), blotted dry and weighed. Subsequently, a blood sample was collected from the caudal blood vessel using a heparinised syringe. Blood was immediately centrifuged (for 1 min at 16,000 rpm at 4 °C), and aliquots of plasma were frozen in liquid nitrogen and stored at –80 °C for later analysis. Fish were dissected on ice, and gills, liver and muscle tissue were removed, frozen in liquid nitrogen and stored at –80 °C for further biochemical analysis and enzymatic assays. In addition, one portion of gills was added to five volumes of RNeasy Lysis Buffer (Qiagen, Hilden, Germany) and stored at 4 °C for later molecular analysis.

2.4. Analytical techniques and calculations

2.4.1. Water sample

Water total ammonia was determined colorimetrically by using the salicylate–hypochlorite method (Verdouw et al., 1978) and urea concentrations by the diacetyl monoxime assay (Rahmatullah and Boyde, 1980).

2.4.2. Plasma metabolites

Ammonia levels in plasma were determined using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany). Plasma lactate concentrations were analysed enzymatically using a commercially available kit (R-Biopharm AG, Darmstadt, Germany). Plasma $[\text{Na}^+]$, $[\text{Cl}^-]$ and $[\text{K}^+]$ were analysed using an AVL 9180 Electrolyte Analyzer (AVL, Roche Diagnostics, Belgium). Plasma osmolality was determined by the Micro-Osmometer 3320 (Advanced instruments, Inc, USA).

2.4.3. Tissue energy store

Samples of liver and muscle were analysed for protein content by Bradford's method (Bradford, 1976), glycogen content by using the anthrone reagent (Roe and Dailey, 1966), and lipid content was measured following Bligh and Dyer (1959).

2.4.4. Muscle water content (MWC) and hepatosomatic index (HSI)

Traces of water and blood on the excised muscle were wiped off with tissue paper. MWC was determined as the percentage of weight loss after drying at 100 °C for 2 days.

Whole liver mass was recorded and hepatosomatic index was calculated as $\text{HSI} = (\text{LM}/\text{BW}) \times 100$, where LM is referred as liver mass and BW is the body weight.

2.4.5. Enzymatic analyses

NKA activity and H^+ -ATPase activity were measured in crude gill homogenates using previously published methods (Lin and Randall, 1993; McCormick, 1993), as modified by Nawata et al. (2007). Protein concentrations were measured with Bradford reagent and BSA standards (Sigma).

2.5. Molecular analyses

2.5.1. RNA extraction

Total RNA was isolated from gill samples using Trizol (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. The extracted RNA samples were DNase treated to avoid genomic DNA contamination. The quantity of the RNA was evaluated by using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). The integrity (quality) was checked by using a gel cartridge on a QIAxcel platform (Qiagen, Hilden, Germany) and the purity by measuring the OD260/OD280 absorption ratio (>1.95).

2.5.2. Real-time PCR

For quantitative real-time PCR (qPCR), a starting amount of 1 μg RNA was transcribed into first-strand cDNA using the Revert Aid H minus first-strand cDNA synthesis kit (Fermentas, Cambridge, UK). The mRNA expression of Na^+/K^+ -ATPase alpha-subunit 1 (α -subunit1) and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter 1 (NKCC1) in the gills was quantified by qPCR using the specific primers listed in Table 1.

qPCR analyses were performed on an Mx3000P QPCR System (Agilent Technologies, Belgium). Reactions containing 5 μL of 5 $\times$ diluted cDNA, 10 pmol each of forward and reverse primers, 0.4 μL ROX dye (1:500 dilution) and 10 μL Brilliant II SYBR Green qPCR (Agilent) were performed in a four-step experimental run protocol: a denaturation program (10 min at 95 °C), an amplification and quantification program repeated 40 times (30 s at 95 °C, 50 s at 55 °C, 40 s at 72 °C), a melting curve program (55–95 °C with a heating rate of 0.10 °C/s and a continuous fluorescence measurement) and finally a cooling step. Melt curve analyses of the target genes and reference genes resulted in single products with specific melting temperatures. In addition, 'no-template' controls (i.e., with water sample) for each set of genes were also run to ensure no contamination of reagents, no primer–dimer formation, etc.

Comparison of two reference genes (elongation factor-1 α and 18 s rRNA) favoured elongation factor-1 α (EF-1 α) as the most stable gene across the samples (20 random samples were tested) and was used as endogenous standard to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples.

2.6. Statistical analysis

All data have been presented as mean values $\pm$ standard error (SE). For comparisons between different experimental groups, a one-way analysis of variance (ANOVA) was performed followed by the least significant difference (LSD) test. Student's two-tailed t -test was used for single comparisons. Main effects of salinity and feeding status and their interactions were analysed by two-way ANOVA. The data were analysed by Statistical Package for the Social Sciences (SPSS) version 20.0. A probability level of 0.05 was used for rejection of the null hypothesis.

3. Results

3.1. Ammonia and urea excretion rate

Ammonia excretion rate (J_{Amm}) declined with decreasing salinity in fasted fish and was significantly inhibited when acclimated to the lowest salinity (2.5 ppt) (Fig. 1A). The relative J_{Amm} in fasted fish acclimated to

Table 1
qPCR primer sequences, accession numbers and calculated efficiency.

Gene	Primer sequence (5' → 3')	Efficiency	References	Accession no.
Na ⁺ K ⁺ -ATPase (α-subunit1)	Forward: CTGGAGTGGAGAAGGTC Reverse: GATGAAGAGGAGGAAGG	108%	Giffard-Mena et al. (2008)	AY532637
Na ⁺ /K ⁺ /2Cl ⁻ cotransporter 1	Forward: TCATCACTGCTGGAATCTT Reverse: AGAGAAACCCACATGTTGTA	91%	Lorin-Nebel et al. (2006)	AY954108
EF 1α	Forward: GCTTCGAGGAAATCACCAG Reverse: CAACCTTCCATCCCTTGAAC	98%	Geay et al. (2010)	AJ866727
18S rRNA	Forward: CGCTAGAGGTGAAATCTTGGA Reverse: GATCAGATACCGTCGTAGTTCC	112%	Hakim et al. (2009)	AM419038

The accession number refers to the registered sequence from GenBank. F, forward; R, reverse.

2.5 ppt was 37% ($P < 0.01$) lower than its control (32 ppt). However, fed fish at lower salinities were able to retain the J_{Amm} at the control level. The effect of nutritional status was also seen between feeding treatments at 2.5 ppt exposure where J_{Amm} was 30% ($P < 0.05$) lower in fasted fish compared to fed fish.

The effect of salinity on urea excretion rate (J_{urea}) was not significant ($P > 0.05$), either in fed or fasted sea bass (Fig. 1B). However, an increasing trend ($P > 0.05$) was noted for both fed and fasted fish with reducing salinity.

3.2. Plasma metabolites

3.2.1. Ammonia and urea accumulation

The effects of feeding as well as salinity challenge were apparent in plasma total ammonia (T_{Amm}) level (Fig. 2A). T_{Amm} was significantly elevated by 59% ($P < 0.01$) in fasted fish at 10 ppt compared to their control at 32 ppt. This effect was intensified when fasted fish were reared in 2.5 ppt, with a 126% increment ($P < 0.01$). Fed fish followed the same pattern as fasted fish; however, the increase was only

Fig. 1. Ammonia excretion rate (A) and urea excretion rate (B) in fed and fasted fish during acclimation to different salinities. Values are mean $\pm$ SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (** $P < 0.01$), and dragger (†) indicates a significant difference between fed and fasted fish at the same salinity (* $P < 0.05$).

Fig. 2. The accumulation of total ammonia (A) and urea (B) in the plasma of fed and fasted fish during acclimation to different salinities. Values are mean $\pm$ SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (* P < 0.05; ** P < 0.01), and dragger (†) indicates a significant difference between fed and fasted fish at the same salinity († P < 0.05; †† P < 0.01).

significant at 2.5 ppt. A difference between feeding treatments was noted for 10 ppt and 2.5 ppt groups; T_{amm} for fasted fish was significantly higher than the corresponding values for fed fish by 61% (P < 0.01) and 52% (P < 0.05), respectively. Overall, a significant interaction (Table 4) was observed between salinity and feeding status on plasma ammonia levels of fish.

Despite the remarkable increase in plasma ammonia level during the salinity challenge, little change (P > 0.05) in the urea concentration in

plasma was observed for both fed and fasted fish in response to salinity change (Fig. 2B).

3.2.2. Lactate

Acclimation to the lower salinities induced plasma lactate accumulation in feed-deprived fish, and the effect was significant in fish acclimated to 10 ppt (31% increment, P < 0.05) and 2.5 ppt (36% increment, P < 0.05) relative to the control (Fig. 3). In fed fish, plasma lactate level remained

Fig. 3. Lactate content in the plasma of fed and fasted fish during acclimation to different salinities. Values are mean $\pm$ SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (* P < 0.05).

fairly constant. No obvious differences ($P > 0.05$) between fed and fasted fish were reported in any of the salinity treatment.

3.2.3. Osmolality

Plasma osmolality among fed fish adapted to the different salinities remained unchanged ($P > 0.05$) throughout the experiment (Fig. 4). However, relative to the 32 ppt control group, osmolality was notably lowered in fasted fish reared at 10 ppt and 2.5 ppt, respectively, with a 18% ($P < 0.01$) and 23% ($P < 0.01$) reduction.

3.2.4. Ions

Plasma $[Na^+]$ responded to salinity change in the same way as plasma osmolality; a significant reduction was noted in the fasted fish acclimated to 10 ppt and 2.5 ppt (Fig. 5A). However, a completely different pattern was seen for $[Cl^-]$ when fish were held at these two salinities (10 ppt and 2.5 ppt), and a significant increase was evident during feed withdrawal (Fig. 5B). No prominent effect of salinity regime or feeding treatment was evident on $[K^+]$ in plasma (Fig. 5C). Lowering environmental salinity resulted in the drop of plasma $[Na^+]:[Cl^-]$ ratio in both fed and fasted fish compared to their respective control (Table 2). However, such reductions became significant ($P < 0.001$) only in the fasted group when acclimated at 10 ppt and 2.5 ppt. At these two salinities, values in the fasted fish were also significantly lower compared to the respective fed groups.

3.3. Tissue metabolites

3.3.1. Energy stores

Glycogen content in the liver of fed fish remained stable in all the salinities but in fasted fish glycogen content decreased during acclimation to 10 ppt and 2.5 ppt, respectively, by 24% ($P < 0.01$) and 30% ($P < 0.01$) compared to the control (Table 3). A difference ($P < 0.001$) between the two feeding regimes was seen at 20 ppt, 10 ppt and 2.5 ppt. In muscle glycogen, fasted fish showed a significantly lower glycogen content than the control group only at 2.5 ppt salinity (Table 3). Overall, the effects of salinity ($P < 0.05$) and food ($P < 0.001$) individually as well as combined ($P < 0.01$) were significant for liver glycogen content (Table 4).

Analogous to the glycogen store, a significant drop in the lipid content was seen in the liver of fasted fish when reared at 2.5 ppt salinity (Table 3). The value reduced by 31% ($P < 0.05$) and 39% ($P < 0.01$), respectively, compared to the control and the parallel group of fed fish. An almost identical trend was evident for HSI (Table 2). Lipid stores in muscle did not change in any experimental group.

3.3.2. Muscle water content (MWC)

MWC in both fed and fasted gradually increased with the reducing salinity of the rearing medium, but in fed fish, MWC was never significantly different from the control value (Table 2). On the contrary, in fasted fish acclimated to 2.5 ppt, the MWC increased significantly from a control value of 70.5% to 85%.

3.4. Activity of NKA and H^+ -ATPase in gill tissue

NKA activity in the gill of fed fish adapted to 10 ppt and 2.5 ppt was significantly higher than in those reared at normal seawater (32 ppt) (Fig. 6A). The relative increment at 10 ppt was 62% ($P < 0.05$) higher than the control, and a further enhancement to 83% ($P < 0.01$) was noted for the 2.5 ppt acclimated group. Analogous to the fed fish, an increasing trend was also noted for fasted fish at lower salinities, but it remained statistically insignificant.

Branchial H^+ -ATPase activity could not be detected in both feeding treatments when acclimated to 32 ppt, 20 ppt and 10 ppt (Fig. 6B). However, the activity was perceptible when fish were reared at the lowest salinity (2.5 ppt).

3.5. Gene expression of NKA and NKCC

Significant effects of salinity changes were seen on NKA mRNA expression levels in gills of fed fish only (Fig. 7). In fed fish, NKA expression at 10 ppt and 2.5 ppt salinities increased respectively by 1.48-fold ($P < 0.01$) and 1.70-fold ($P < 0.01$) compared to the control. In contrast, unfed fish did not show remarkable change in function to the salinity challenge. In general, the response of NKA activity paralleled mRNA expression data quite well for both feeding treatments.

Branchial NKCC expression in both fed and fasted fish decreased when subjected to lower salinities (Fig. 8). A 1.81-fold reduction ($P < 0.05$) was observed for fasted individuals acclimated to 10 ppt in comparison to control. A more pronounced reduction (2.75-fold, $P < 0.01$) was evident when fasted fish were reared at 2.5 ppt. The significant effect of salinity for fed fish was seen only at the lowest salinity (2.5 ppt) with a 1.56-fold ($P < 0.05$) reduction relative to the control. The difference between feeding treatments was seen when fish were acclimated to 2.5 ppt salinity, and the mRNA transcript level in fasted fish was significantly lower (1.53 fold) than that in the fed ones.

The interaction between the two experimental factors (salinity exposure and feeding regimes) for all the different parameters investigated in the present study has been illustrated in Table 4.

Fig. 4. Plasma osmolality of fed and fasted fish during acclimation to different salinities. Values are mean $\pm$ SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (** $P < 0.01$).

Fig. 5. Concentration (mmol/L) of (A) Na⁺ (B) Cl⁻ and (C) K⁺ in the plasma of fed and fasted fish during acclimation to different salinities. Values are mean $\pm$ SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (* P < 0.05; ** P < 0.01; *** P < 0.001), and dragger (†) indicates a significant difference between fed and fasted fish at the same salinity († P < 0.05; †† P < 0.01).

Table 2

Hepatosomatic index (HSI), muscle water content (MWC) and [Na⁺]/[Cl⁻] ratio in European sea bass under different treatments.

	32 ppt		20 ppt		10 ppt		2.5 ppt	
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
HSI	3.64 $\pm$ 0.22	3.39 $\pm$ 0.22	3.88 $\pm$ 0.54	3.20 $\pm$ 0.29	3.59 $\pm$ 0.34	2.97 $\pm$ 0.28	3.25 $\pm$ 0.27	2.46 $\pm$ 0.19**†
MWC	72.00 $\pm$ 4.03	70.50 $\pm$ 4.52	69.88 $\pm$ 3.63	73.25 $\pm$ 3.20	80.13 $\pm$ 3.07	81.00 $\pm$ 4.17	82.63 $\pm$ 3.29	84.88 $\pm$ 3.94*
[Na ⁺]/[Cl ⁻]	1.434 $\pm$ 0.12	1.426 $\pm$ 0.09	1.406 $\pm$ 0.14	1.283 $\pm$ 0.13	1.093 $\pm$ 0.10	0.831 $\pm$ 0.06***†	1.055 $\pm$ 0.11	0.757 $\pm$ 0.05***††

Values are mean $\pm$ SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (* P < 0.05, ** P < 0.01, *** P < 0.001), and dragger (†) indicates a significant difference between fed and fasted fish at the same salinity († P < 0.05, †† P < 0.01).

Table 3

Energy store in liver and muscle of European sea bass under different treatments.

	32 ppt		20 ppt		10 ppt		2.5 ppt	
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
<i>Glycogen content (mg/g wet tissue)</i>								
Liver	152.4 ± 7.91	139.0 ± 7.71	168.0 ± 6.87	120.5 ± 7.75 ^{†††}	171.1 ± 5.72	106.2 ± 8.7 ^{***†††}	155.8 ± 5.46	97.6 ± 8.16 ^{***†††}
Muscle	1.56 ± 0.06	1.39 ± 0.08	1.49 ± 0.07	1.34 ± 0.07	1.44 ± 0.07	1.26 ± 0.10	1.31 ± 0.09	1.14 ± 0.07 [*]
<i>Lipid content (mg/g wet tissue)</i>								
Liver	28.2 ± 2.28	25.7 ± 1.58	30.5 ± 3.09	28.8 ± 2.79	32.3 ± 3.28	24.8 ± 2.48	29.1 ± 2.72	17.9 ± 2.02 ^{*††}
Muscle	16.3 ± 1.75	18.9 ± 1.55	15.5 ± 1.77	16.0 ± 2.20	20.1 ± 2.13	15.5 ± 1.49	14.0 ± 1.90	13.0 ± 1.58
<i>Protein content (mg/g wet tissue)</i>								
Liver	252.8 ± 15.5	277.0 ± 17.4	258.5 ± 16.3	296.6 ± 9.86	288.8 ± 20.4	309 ± 17.4	285.3 ± 16.9	323.9 ± 25.1
Muscle	271.7 ± 12.8	281.1 ± 18.9	282.2 ± 18.5	264.0 ± 19.3	268.7 ± 17.7	276.5 ± 16.0	274.3 ± 15.6	286.5 ± 18.5

Values are mean ± SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (* $P < 0.05$; ** $P < 0.01$), and dragger (†) indicates a significant difference between fed and fasted fish at the same salinity († $P < 0.05$; †† $P < 0.01$ and ††† $P < 0.001$).

4. Discussion

4.1. Osmoregulatory adjustments

The present study suggests that plasma osmolalities of euryhaline teleosts (at least in sea bass) are not merely influenced by the salinity fluctuation reported earlier (Kato et al., 2005; Bystriansky et al., 2006; Kang et al., 2008) but also by the nutritional status (Polakof et al., 2006). Osmolality level in the plasma of fed fish acclimated to lower salinities was maintained within the range of control values (32 ppt), suggesting an efficient hypo-osmoregulation capacity. However, in the fasted fish, the osmolality dropped by approximately 20–25% at lower salinities reflecting an osmotic perturbation, which might be associated with a constraint in the adaptation process. The composition as well as the content of the extracellular fluid determines the competency of euryhaline teleosts to tolerate changes in salinity (Freire et al., 2008). Therefore, in the course of acclimation to the varying osmolalities, the water content must be efficiently regulated in the body to achieve

water balance. Muscle water content (MWC) is widely used as a physiological index to evaluate the water balance in fish; a change in MWC levels may be an imperative indicator of osmoregulatory capacity and degree of euryhalinity (Freire et al., 2008; Tang et al., 2009). MWC of seawater medaka (*Oryzias dancena*) remained stable after acclimation to a freshwater environment (Kang et al., 2008). Likewise, the emperor angelfish (*Pomacanthus imperator*) exhibited constant levels of MWC upon a salinity challenge (Woo and Chung, 1995). The black sea bream (*Mylio macrocephalus*) (Kelly et al., 1999) and gilthead sea bream (*Sparus aurata*) (Kelly and Woo, 1999) had an augmented MWC after transfer from marine to low salinity habitat. Our results revealed that after acclimation to low salinity (2.5 ppt), MWC values in fasted fish increased considerably by 21% compared to that at 32 ppt. The sturdy increase in MWC among fasted fish might have been a consequence of reduction in muscle lipid and glycogen content (Table 3) as was also suggested in other studies (*D. labrax*, Alliot et al., 1984; Jensen et al., 1998; *S. salar*, Saunders and Henderson, 1978) and/or is likely related to the enhanced osmotic water entrance, which also explains the lower blood osmolality. Meanwhile, the MWC of the fed groups remained stable in all the tested salinities, reflecting a better potential to maintain the water balance. Thus, feeding certainly ameliorates osmoregulatory acclimation in sea bass and facilitates rapid adaptation to the new environment.

4.2. Ion-regulatory response

Salinity challenge induces intricate ion-regulatory changes in the gills of marine teleosts (Jensen et al., 1998; Lin et al., 2003; Giffard-Mena et al., 2008; Kang et al., 2008). Gill NKA activity is associated with the secretion of excess ions in a hyperosmotic environment (McCormick, 2001; Marshall, 2002) and ion uptake in a hypo-osmotic environment (Jensen et al., 1998; Lin et al., 2004a). The activity of NKA was significantly activated in response to lower salinities (10–2.5 ppt), possibly as a compensatory mechanism to counteract ion loss induced by low salinity media. It has previously been reported that in European sea bass reared at lower salinities (close to freshwater), branchial NKA was elevated (Lasserre, 1971). Likewise, gill NKA activity was reported to be higher in freshwater than in the seawater-acclimated milkfish (*Chanos chanos*) (Lin et al., 2003), thicklip grey mullet (*Chelon labrosus*) (Lasserre, 1971; Gallis et al., 1979), Australian bass (*Maquaria novemaculata*) (Langdon, 1987), black seabream (*M. macrocephalus*) (Kelly et al., 1999) and killifish (*Fundulus heteroclitus*) (Katoh et al., 2002).

The ion-regulatory mechanism involving NKA is driven by energy. Thereby, the depletion in energy store observed (Table 3) in unfed fish might have limited the activation, as only the fed fish seemed able to activate branchial NKA sufficiently. Similarly, branchial NKA activity was elevated in fed gilthead sea bream acclimated to lower salinity while food deprivation for 14 days abolished such increment (Polakof

Table 4

The effects of salinity acclimatisation and feeding status and their interactions on the metabolic, physiological and ion-osmoregulatory parameters investigated in the sea bass.

Treatments	Salinity		Feeding		Salinity × Feeding	
	F value	P value	F value	P value	F value	P value
<i>Nitrogen excretion</i>						
Ammonia excretion	6.091	0.001	10.256	0.002	0.500	0.684
Urea excretion	0.388	0.763	0.360	0.551	0.189	0.903
<i>Plasma metabolites</i>						
Ammonia	12.139	0.000	8.113	0.006	3.493	0.022
Urea	1.317	0.280	0.138	0.712	0.025	0.995
Lactate	2.977	0.039	3.726	0.059	0.853	0.471
Osmolality	7.734	0.000	1.027	0.315	0.622	0.604
Na ⁺	7.071	0.000	6.081	0.017	0.306	0.821
Cl [−]	5.876	0.002	1.037	0.313	0.727	0.540
K ⁺	4.588	0.007	0.062	0.804	0.052	0.984
Na ⁺ :Cl [−] ratio	7.071	0.000	6.081	0.017	0.306	0.821
<i>Branchial ion transporters</i>						
Na ⁺ /K ⁺ -ATPase activity	5.049	0.004	3.135	0.083	0.759	0.523
Na ⁺ /K ⁺ -ATPase expression	3.964	0.013	1.004	0.321	1.625	0.195
Na ⁺ /K ⁺ /2Cl [−] expression	6.539	0.001	1.548	0.219	0.978	0.410
<i>Liver metabolites</i>						
Glycogen	2.748	0.041	77.808	0.000	4.817	0.005
Lipid	2.032	0.121	9.088	0.004	1.357	0.266
Protein	2.155	0.104	5.743	0.020	0.142	0.935
Hepatosomatic index	2.068	0.115	7.127	0.010	0.285	0.836
<i>Muscle metabolites</i>						
Glycogen	3.393	0.024	8.523	0.005	0.008	0.999
Lipid	2.327	0.085	0.237	0.629	1.337	0.272
Protein	0.018	0.997	0.001	0.970	0.255	0.858
Muscle water content	5.684	0.002	0.221	0.640	0.156	0.926

Fig. 6. Enzyme activity of (A) Na⁺/K⁺-ATPase and (B) H⁺-ATPase in the gills of fed and fasted fish during acclimation to different salinities. Values are mean ± SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (**P* < 0.05; ***P* < 0.01).

et al., 2006). It confirms that feeding facilitates the acclimation mechanisms during salinity stress, rendering energy available from food (Sucré et al., 2013). The increase in NKA activity among fed fish was accompanied by a parallel activation of the transcript level of NKA-α1, the predominant isoform in gills of marine teleosts (Giffard-Mena

et al., 2008), which was not seen in fasted fish. Similarly, Hakim et al. (2009) reported a decline in mRNA expression of this enzyme when European sea bass were fasted for 14–21 days. Furthermore, this basolaterally situated NKA is believed to provide the major source of energy driving Na⁺ influx from the external water into the blood (see

Fig. 7. Expression of Na⁺/K⁺-ATPase mRNA in the gills of fed and fasted fish during acclimation to different salinities. Values are mean ± SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (***P* < 0.01).

Fig. 8. Expression of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter 1 mRNA in the gills of fed and fasted fish during acclimation to different salinities. Values are mean $\pm$ SE. Asterisk (*) indicates a significant difference between the salinity-acclimated fish and its respective control (at 32 ppt) (* $P < 0.05$; ** $P < 0.01$), and dagger (†) indicates a significant difference between fed and starved fish at the same salinity († $P < 0.05$).

introduction). The enhanced activity in fed fish aided to regulate their ion status, evident by the retention of Na^+ at the control level even in a hypo-osmotic environment. In freshwater fish, beside the potential involvement of Rh glycoproteins and Na^+/H^+ exchangers (NHE), the gradient needed for sodium uptake is also linked to the action of apical H^+ -ATPase (Weihrauch et al., 2009; Wright and Wood, 2009, 2012). However, the functional role of H^+ -ATPase is doubtful in marine teleosts. Interestingly, to our best knowledge, no previous study has examined the changes in the H^+ -ATPase activity following transfer of European sea bass to freshwater. We found that gill H^+ -ATPase was not detected in the seawater (32 ppt) and brackish water (20 ppt and 10 ppt) acclimated fish groups, suggesting that in these environments H^+ -ATPase remains in the dormant phase. However, when holding the fish in a hyposaline environment at 2.5 ppt, the necessity to drive Na^+ uptake tends to increase and H^+ -ATPase was activated. Hence, although European sea bass are not a fully diadromous fish, our result suggests that H^+ -ATPase is present in the branchial cell and can be activated when triggered by the salinity stimulation. Taken together, as salinity decreases, sea bass rely primarily on the NKA empowering Na^+ uptake, whereas the contribution of H^+ -ATPase for ion transportation is only important at very low salinities. Nevertheless, the limitations of this study need to be mentioned, as the relative contribution of Rh glycoprotein and NHE for sodium uptake was not quantified.

Besides NKA, NKCC is an important ion transport protein, predominantly localised on the basolateral side of the chloride cells in marine teleosts (Wilson et al., 2000; Marshall et al., 2002; Lorin-Nebel et al., 2006) and is proposed to be involved in ion secretion (Lorin-Nebel et al., 2006). NKCC facilitates the uptake of Na^+ , Cl^- and K^+ from blood into the cell (Marshall, 2002), and this co-transportation is mediated by the electrochemical gradient created by the NKA. Low salinity acclimation in sea bass induced a down-regulation of NKCC mRNA expression also reported by Lorin-Nebel et al. (2006). This response at lower salinities probably enables sea bass to rapidly decrease net ion secretion. Furthermore, a further reduction in transcript level of NKCC mRNA among fasted fish (compared to fed fish) was concomitant with the increase in plasma $[\text{Cl}^-]$. It suggests that branchial secretion mechanisms are depressed to a larger extent by fasting resulting in a high accumulation of plasma $[\text{Cl}^-]$. In addition, Na^+/Cl^- cotransporters (NCC) are involved in Cl^- uptake from the external environment (Hsu et al., 2014). Although not measured in the present study, it is reasonable to speculate that elevated plasma $[\text{Cl}^-]$ may also relate to the higher expression of NCC during starvation. Also, the more pronounced alteration in NKCC expression in the fasted

fish may be a compensation for the non-incremental response of the NKA.

Salinity-induced alterations in the circulating electrolytes and plasma osmolality of euryhaline teleosts are well acknowledged. Similar to present finding, in most marine teleosts plasma Na^+ and Cl^- respond to change in ambient salinity in the same way as they respond to osmolality (Mancera et al., 1993; Ciccotti et al., 1994; Gaumet et al., 1995; Woo and Chung, 1995; Jensen et al., 1998; Kelly et al., 1999). Moreover, Na^+ and Cl^- are the dominating ions in the blood, and a change in their ratio is recommended as an estimate of acid-base imbalance or blood pH. Madsen et al. (1996) and Maxime et al. (1991) proposed that a relative increment in plasma $[\text{Cl}^-]$ compared to $[\text{Na}^+]$ is associated with a decline in plasma $[\text{HCO}_3^-]$ resulting a drop in blood pH. Consequently, in the current study a non-significant drop in the plasma $[\text{Na}^+]:[\text{Cl}^-]$ ratio in fed fish after low salinity acclimation suggest a very mild and momentary metabolic acidosis. On the contrary, unfed fish reared at lower salinities seems to experience a severe metabolic acidosis as evident by a significant drop in $[\text{Na}^+]:[\text{Cl}^-]$ ratio. Despite the fact that a direct measurement of blood pH was not measured, this corresponds well with the concomitant increase in plasma lactate, suggesting metabolic acidosis in fasted fish.

4.3. Energy store and expenditure

Energy supply is prerequisite for the operation of ion-osmoregulatory mechanism in fish. There is general consensus that carbohydrate oxidation is the major metabolic fuelling source for ion- and osmoregulation. Liver is the central site involved in glycogen mobilisation and supplying carbohydrate metabolites to osmoregulatory organs (for a review, see Tseng and Hwang, 2008). Therefore, it is obvious that during osmotic adaptation liver metabolism will be enhanced to serve glucose as energy source for the operation of various biochemical processes in osmoregulatory organs such as gills. In the present study, liver glycogen levels decreased considerably in unfed fish exposed to low salinities compared to its seawater control as well as to the respective fed groups, suggesting that acclimatising feed-deprived sea bass to lower salinities induces glycogenolysis at a faster rate for osmoregulation; while in fed fish glycogenesis and glycogenolysis remained in steady state. Probably the reduced liver glycogen stores in fasted fish might have hampered energy-reliant NKA functioning, resulted in a limited activation compared to fed fish.

Fasted fish clearly had a smaller hepatosomatic index, indicating that liver energy stores such as glycogen and lipids had been used.

However, neither fasted nor fed fish mobilised protein store as the fuel for tissues during osmotic acclimation. On the contrary, the mild increase in liver protein in fasted fish relative to fed fish and seawater controls is striking. Increased liver protein level has also been reported in rainbow trout (*Salmo gairdneri*) (Jurss, 1979) and tilapia (Jurss et al., 1984) after feed deprivation and salinity stress. Such increment in protein level is hypothesised to provide a protective mechanism against osmotic perturbation when glycogen content reduces extensively (Jurss et al., 1984). This is also supported by the present study where significant reduction in glycogen content was recorded during starvation.

The increase in plasma lactate in fasted fish highlights the onset of anaerobic metabolism, despite the normoxic environment, and suggests that this metabolite may be used as energy fuel for osmotic adaptation during long term starvation. Our result also indicates that fed fish have the ability to cover the ion-osmoregulatory expenses by aerobic metabolism as reflected by the lack of plasma lactate accumulation. Plasma lactate accumulation seems to be supported by the mobilisation of liver glycogen; the production of lactate in fasted fish correlates with the decreased glycogen store. Similar to the present finding, the acclimation of gilthead sea bream to extreme salinities enhanced activity of osmoregulatory organs, which was sustained by higher glucose supply in fed fish but by increased use of other fuels, such as lactate in food-deprived individuals (Polakof et al., 2006).

The possible signalling mechanism between metabolic status and the osmoregulatory response of the fish during osmotic adaptation are not yet fully understood. In this context, it is reasonable to speculate that hormones such as cortisol, prolactin, growth hormone and thyroid hormones controlling ion-osmoregulation and metabolic processes may be involved. Therefore, investigation of these hormones may be crucial in future related studies.

4.4. Ammonia and urea dynamics

Ammonia and urea kinetics permit to evaluate the nitrogen balance and are also used as indicators for assessing the effect of environmental factors on protein catabolism. Information pertaining to salinity effects on nitrogen waste metabolism among piscine group is somewhat scarce and discordant, probably due to species-specific differences in salinity adaptation. We found that fasted fish were not able to excrete ammonia sufficiently when acclimated to 2.5 ppt, which was also reflected by the more than 2-fold elevation of plasma T_{Amm} relative to the control (32 ppt). Fed fish appear to regulate ammonia homeostasis more efficiently. The lower J_{Amm} in the fasted fish relative to the fed fish could also indicate that protein usage was higher in the fed fish, presumably due to the breakdown of protein from dietary intake rather than tissue store as these fish retained liver and muscle protein stores to the basal level. As such, the low ammonia excretion rates of the fasted fish might represent the endogenous or maintenance fraction (Alsop and Wood, 1997) and differences between the fed and fasted fish (during 2.5 ppt) could be attributed to the exogenous fraction or the portion not retained from the absorbed food. Ingested proteins are hydrolysed into amino acids, which are then absorbed and utilised for protein synthesis. Excess amino acids in protein metabolism are broken down, leading to high ammonia production. This ammonia is then excreted, either by diffusion of ammonia or as the ammonium ion. NH_4^+ is moved across the apical membrane in loose exchange for apical Na^+ uptake (probably by NHE-3). Therefore, the increased NKA activity noted for the fed fish would have intensified the driving force to take up Na^+ from the water and subsequently as a part of exchange process triggered ammonia efflux out of the fish (Avella and Bornancin, 1989; Wilkie, 1997; Patrick and Wood, 1999; Randall and Tsui, 2002). Furthermore, there are reports of effective NH_4^+ substitution for K^+ on the NKA in the gills of several euryhaline and marine fish (Mallery, 1983; Balm et al., 1988; Randall et al., 1999; Nawata et al., 2010), suggesting that an increase in NKA activity would induce ammonia excretion,

reinforcing our finding for the fed fish. However, the potential ability of NH_4^+ to activate gill NKA was not evaluated in the current study. Since plasma T_{Amm} levels in fasted fish were considerably higher, the diffusion gradient for ammonia excretion is likely to be more favourable in fasted compared to fed fish, and excretion should have been easier. This indicates that other mechanisms played a role resulting in a more efficient excretion process in fed fish. Perhaps, it is related to the Rh glycoprotein expression in fish gill (Hung et al., 2007; Nakada et al., 2007; Nawata et al., 2007, 2010; Weihs et al., 2009; Wright and Wood, 2009, 2012). Rh glycoproteins present in the gill cell membranes are implicated as a putative mechanism of ammonia transport. Studies in freshwater fish have shown that feeding triggers an upregulation of Rh mRNA expression (Zimmer et al., 2010), resulting in higher ammonia excretion rate in fed fish. However, molecular evidence of Rh glycoproteins (and NHE-3) in sea bass is at present still lacking.

Ammonia is either excreted directly if feasible or converted to some less toxic compound such as urea. Although the majority of teleost fishes are ammoniotelic, urea also constitutes about 10–30% of the total nitrogenous wastes in most of them (Saha and Ratha, 1998). Data presented in our study indicate that there was no major disturbance in plasma urea, and urea excretion rate was not affected by the salinity fluctuation. It supports the earlier finding in European sea bass revealing no induction in urea synthesis or excretion in response to environmental stressors (Dosdat et al., 2003). Likewise, in the same fish species, no variation in urea concentration was noted in plasma during 50 days of chronic starvation (Echevarria et al., 1997). Ureogenesis in fishes is energetically expensive; the production of urea and its subsequent excretion would result in a high expenditure of energy and the loss of carbon. Probably, because of these drawbacks ureogenesis is not a common mechanism adopted by sea bass to cope with adverse conditions. The source of urea production in marine teleosts and the involvement of the ornithine urea cycle are still doubtful. It would be interesting to investigate the involvement of urea transporter (UT) in facilitating the diffusion of urea across branchial epithelium. Although some studies have been conducted in freshwater fish (Wood and Nawata, 2011; Sinha et al., 2013), the function of UT in marine teleosts such as sea bass is yet to be evaluated.

5. Conclusion

The results of the present study suggest that the feeding status modulates physiological, biochemical and ion-osmoregulatory responses differentially in fed and fasted European sea bass acclimated to salinity regimes of 10 ppt and 2.5 ppt. Unlike fasted sea bass, fed fish in experimental salinities from full strength seawater (32 ppt) to hyposaline water (2.5 ppt) maintained their osmolality, ionic concentration and muscle water content at the same level. It suggests that fed fish exhibited better hypo-osmoregulatory ability than fasted ones, which is supported by an increment in the activity and the expression of NKA. Furthermore, as a compensatory response to maintain constant blood osmolality, a reduction in NKCC mRNA transcript in both feeding treatments might enable the fish to rapidly decrease net ion secretion during acclimatisation to lower salinities (10 ppt and 2.5 ppt). Interestingly, H^+ -ATPase was expressed only at the lowest salinity (2.5 ppt), probably assisting the fish for Na^+ uptake from the hyposaline media. Our findings also indicate that feed restriction exhibited reorganisation of energy metabolism in liver and muscle tissue, manifested by the depletion of glycogen and lipid reserve in fasted fish. In addition, the reduction of glycogen in feed-deprived fish was concomitant with an increase in plasma lactate level, indicating a switch to anaerobic metabolism during aerobic fuel shortage. During hypo-osmotic acclimation, ammonia excretion was inhibited in fasted fish with a considerable increment in plasma ammonia level. On the contrary, fed fish were able to excrete ammonia efficiently. In brief, fed fish have an advantage over food-deprived individuals, and food deprivation reduces the physiological, metabolic and ion-regulatory performance of fish when

farmed in low ambient saline water. For aquaculture and farm management, it can be advised to transfer sea bass to isotonic water whenever the threat of starvation is anticipated, but this proposal has yet to be evaluated in economic terms. Our study also provides a cautionary note as to the importance of considering feeding as a crucial factor in salinity stress studies.

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