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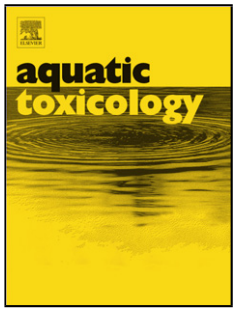

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Interactive effect of high environmental ammonia and nutritional status on ecophysiological performance of European sea bass (*Dicentrarchus labrax*) acclimated to reduced seawater salinities

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Abstract

We investigated the interactive effect of ammonia toxicity, salinity challenge and nutritional status on the ecophysiological performance of European sea bass (*Dicentrarchus labrax*). Fish were progressively acclimated to normal seawater (32 ppt), to brackish water (20 ppt and 10 ppt) and to hyposaline water (2.5 ppt). Following acclimation to different salinities for two weeks, fish were exposed to high environmental ammonia (HEA, 20 mg/L ~ 1.18 mM representing 50% of 96h LC_{50} value for ammonia) for 12 h, 48 h, 84 h and 180 h, and were either fed (2% body weight) or fasted (unfed for 7 days prior to HEA exposure). Biochemical responses such as ammonia (J_{amm}) and urea excretion rate, plasma ammonia, urea and lactate, plasma ions (Na^+, Cl^- and K^+) and osmolality, muscle water content (MWC) and liver and muscle energy budget (glycogen, lipid and protein), as well as branchial Na^+/K^+-ATPase (NKA) and H^+-ATPase activity, and branchial mRNA expression of NKA and $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) were investigated in order to understand metabolic and ion- osmoregulatory consequences of the experimental conditions. During HEA, J_{amm} was inhibited in fasted fish at 10 ppt, while fed fish were still able to excrete efficiently. At 2.5 ppt, both feeding groups subjected to HEA experienced severe reductions and eventually a reversion in J_{amm}. Overall, the build-up of plasma ammonia in HEA exposed fed fish was much lower than fasted ones. Unlike fasted fish, fed fish acclimated to lower salinities (10 ppt-2.5 ppt) could maintain plasma osmolality, $[Na^+]$, $[Cl^-]$ and MWC during HEA exposure. Thus fed fish were able to sustain ion-osmotic homeostasis which was associated with a more pronounced up-regulation in NKA expression and activity. At 2.5 ppt both feeding groups activated H^+-ATPase. The expression of NKCC1 was down-regulated at lower salinities in both fed and fasted fish, but was upregulated within each salinity after a few days of HEA exposure. Though an increment in plasma lactate content and a decline in energy stores were noted for both feeding regimes, the effect was more severe in feed deprived fish. Overall, several different physiological processes were disturbed in fasted sea bass during HEA exposure while feeding alleviated adverse effects of high ammonia and salinity challenge. This suggests that low food availability can render fish more vulnerable to external ammonia, especially at reduced seawater salinities.

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Keywords: Marine teleosts, osmotic stress, ammonia excretion rate, fasting, ionoregulation, osmoregulation, energy metabolism.

1. Introduction

Worldwide, there is a growing concern about the future shift in seawater salinity (IPCC 2013). In the last two decades as a consequence of global warming, glaciers and ice caps have been rapidly disappearing with more frequent intense rainfall events. Consequently, the salinity gradient of some marine ecosystems such as enclosed bays, estuaries, coastal lagoons, and the inshore water has gradually reduced (Freeland and Whitney, 2000; Pierce et al., 2012; Wong et al., 1999). These aquatic systems also experience salinity fluctuations under the impact of tidal and seasonal changes. Beside the conventional salinity anomalies, euryhaline teleosts including diadromous and non-diadromous fish also encounter osmotic challenges at different stages of their life cycle as part of migration, spawning and foraging. In general, salinity adaptation by euryhaline teleosts is a complex process involving a cascade of physiological and ion-regulatory responses. The mechanisms of ion-osmoregulation in marine, euryhaline and freshwater teleosts are reasonably well understood (for reviews, see Dymowska et al., 2012; Evans et al, 2005; Hiroi and McCormick, 2012) which are coordinated by ion channels, co-transporters (e.g. $Na^+/K^+/2Cl^-$, Na^+/Cl^-), and energy dependent ATP-ases (e.g. Na^+/K^+-ATPase and H^+-ATPase).

In natural environments, aquatic animals including fish often simultaneously encounter a combination of different abiotic stresses. The effects of salinity reduction do not act in isolation; increasing human pressure including climate change creates a variety of additional deleterious impacts on animals. In natural and culture-based system, ammonia is a common pollutant, resulting from the anthropogenic inputs of particulate nitrogen and organic nitrogen, excretion of cultured animals and mineralization of organic detritus. High ammonia load induces a range of ecotoxicological effects in

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fish, include decrease in growth rate (Dosdat et al., 2003), alteration in energy metabolism (Arillo et al., 1981; Sinha et al., 2012a), disruption of ionic balance (Diricx et al., 2013; Sinha et al., 2012b, 2014; Wilkie, 1997), increased vulnerability to disease, and even mortality. Usually, salinity has a profound effect on ionization equilibrium of (total) ammonia into toxic gaseous (NH_3) and non-toxic ionized (NH_4^+) form. A reduction of 10 units in salinity (at 20°C) would results in approximately 9% increase in NH_3 fraction, and, therefore, higher ammonia toxicity would be expected at lower salinity (Khoo et al., 1977; Thurston et al., 1979). However, little information is available on the toxic effect of ammonia at different ambient salinities in marine teleosts (Bianchini et al., 1996; Sampaio et al., 2002; Weirich et al., 1993; Weirich and Riche, 2006; Wise and Tomasso, 1989). In addition, ion regulation in fish gills are closely associated with ammonia excretion pathways (Wilkie, 1997, 2002). Basolateral transporters in branchial epithelium such as Na^+/K^+-ATPase (NKA) and $Na^+/K^+/2Cl^-$ co-transporter (NKCC) are primarily associated with ion transport; but their importance in ammonia excretion has been implicated since similarities in the hydration radius of K^+ and NH_4^+ allow substitution at transport sites (Alam and Frankel, 2006; Randall et al., 1999; Wilkie, 1997). Although the bulk of ammonia transport is thought to be through NH_3 diffusion either directly or through Rhesus proteins (Nakada et al., 2007; Nawata et al., 2007), 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). Hence, we postulated that in marine teleost, the metabolic and ion-osmoregulatory response incited by high environmental ammonia (HEA) may be modulated by salinity fluctuation.

Beside osmotic challenges and ammonia toxicity, food restriction is a natural phenomenon in wild populations of fish especially during reproduction and migration, and also occurs regularly for cultured fish as it is used as a management tool to control water quality and disease (Robinson and Li, 1999). The fish ability to cope with varying environmental salinities and ammonia toxicity is primarily regulated by the activation of ion transport processes which impose energetic regulatory costs. Consequently, nutritional status can have a pronounce impact on the performance, and determine the competency to adapt to changing environments. Earlier studies in teleost has shown that the sub-lethal

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effect of ammonia as well as salinity stress are exacerbated during feed deprivation (Diricx et al., 2013; Rodríguez et al., 2005; Sinha et al., 2012a,b,c; Woo and Murat, 1981). Thus, if salinity challenge and ammonia exposure, which both have negative effect on physiological performance and ion-regulation occur simultaneously with food deprivation, synergistic effect can be expected. It is well anticipated that fish may simultaneously experience a suite of stressors which can act independently or act together to impede the resiliency of fish to pollutants causing adverse effect. Though the majority of the research has focused on the impact of a single stressor on the performance of fish; assessment of such responses when fish are subjected to an assortment of multiple stressors such as salinity reduction, ammonia threat and starvation is rather scarce. Therefore, the present experiment was designed to understand how compensatory mechanisms are modulated in fish resulting from the stress induced by the combined effects of ammonia pollution and feed deprivation under a salinity gradient.

European sea bass (*Dicentrarchus labrax* L.) is a euryhaline teleost, is widely distributed throughout the estuaries and open oceans of Europe and is extensively used for aquaculture. It is therefore of great commercial and ecological importance. They migrate seasonally between the open sea and hyposaline environments such as lagoons/estuaries, thus they are often challenged with hypo-osmotic stress (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 responds to different stressors such as low ambient salinity, high environmental ammonia and feed deprivation at the same time.

Overall, we hypothesized that sea bass would be adversely affected by HEA, and the toxic effect of ammonia would be amplified when fish are subjected to hypo-osmotic environments. We also propose 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 and instigate fish less vulnerable to external ammonia during hypo-osmotic challenge.

In brief, the purpose of this study was to investigate the effects of multiple-stressors encompassing high ambient ammonia (20 mg/L ~ 1.18 mM, represents 50% of 96h LC_{50} value

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expressed as total ammonia at pH 8.1; Person-Le Ruyet et al., 1995) and periods of feed deprivation in European sea bass during acclimation to experimental salinities (32, 20,10 and 2.5 ppt) on the metabolic, physiological and ion-osmoregulatory process that occur as compensatory mechanisms to maintain homeostasis. The findings of the present work will help to identify the key environmental cues whose fluctuation both individually and in combination might threaten the performance of marine teleost, thereby assist to formulate the guidelines for the regulation of certain environmental factors for the sustainability of ecological system as well as aquaculture.

2. Materials and methods

European sea bass (*Dicentrarchus labrax*) juveniles (14–18 g; 10-12 months old) were obtained from Ecloserie Marine (Gravelines, France) and transferred to the University of Antwerp. Fish were maintained in 1000 L tanks, filled with artificial seawater (Meersalz Professional Salt, 32 ppt salt). Thereafter, a total of 600 fish were distributed into twenty four 200 L tanks (n = 25 per tank; 32 ppt) equipped with a recirculating water supply in a climate chamber where temperature was adjusted at 17±1°C and photoperiod was 12 h light and 12 h dark. Fish were acclimated to the above mentioned constant salinity, temperature and photoperiod for one month prior to the experiment and were fed with commercial pellets (Skretting, Boxmeer, The Netherlands) at a rate of 2% on their wet body weight/day. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. During the ammonia exposure, charcoal and lava stones were removed from the filter to prevent ammonia absorption in the filter. Similarly, they were removed from control tanks as well. 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.

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2.1. Hypoosmotic stress- fish acclimation to lowered seawater salinities

Fish in the tanks were progressively acclimated to three experimental salinities: 20 ppt (~ 500 mOsm/Kg, pH 8.17; 6 tanks); 10 ppt (~ 249 mOsm/Kg, pH 8.10; 6 tanks) and 2.5 ppt (~ 69 mOsm/Kg, pH 7.87; 6 tanks). Fish in remaining 6 tanks were maintained at normal seawater salinity 32 ppt (~ 800 mOsm/Kg, pH 8.25). The salinities of 20 ppt and 10 ppt correspond to brackish water, while 2.5 ppt characterizes hyposaline water. Each experimental group was acclimatized to the desired salinity for 2 weeks and was fed daily at a rate of 2% of their wet body weight. Changes in salinity were progressed by reducing the salinity by 5%, each three day until the desired salinity was reached. Experimental salinities were adjusted by diluting artificial seawater with filtered freshwater, and salinity was measured using a hand-held refractometer.

2.2. Experimental groups and ammonia exposure

After being acclimatized at the respective salinities for 2 weeks, feeding was withheld in three of the tanks belonging to each of the salinity regimes. Fasted fish groups were kept unfed 7 days prior to the ammonia exposure, while feeding (2% bodyweight/day) was continued in the respective three parallel tanks. Feeding was adjusted based on the weight and the number of fish remaining in the tank after each sampling period.

In brief, the experimental set up for each of the salinity group consists of four categories: (1) ammonia unexposed (control) fed fish (1 tank), (2) ammonia exposed fed fish (2 tanks), (3) ammonia unexposed (control) starved fish (1 tank) and, (4) ammonia exposed starved fish (2 tanks). Each exposure tank was spiked with the required amount of an NH_4HCO_3 stock solution (Sigma, Germany). A constant concentration of 1.17 ± 0.06 mM of (total) ammonia was maintained throughout the experiment. Ammonia concentrations were measured (using the salicylate-hypochlorite method, Verdouw et al., 1978) each 6 h after the onset of treatment and the concentration of ammonia in the tank was maintained by adding the calculated amount of the NH_4HCO_3 solution. To avoid the microbial breakdown of test chemical and build-up of other waste products, 40–60% of the water was discarded

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twice a week and replaced with fresh water containing the respective amount of ammonia. The salinity was tested and controlled daily by adding clean water (of the appropriate salinity). Water pH was monitored throughout the experimental period using a pH electrode (Hamilton Bonaduz AG, Metrohm) and was maintained constantly at 8.1.

Ammonia exposed groups for both fed and fasted group acclimated to experimental salinities were sampled after 12 h, 48 h, 84 h and 180 h. Following each exposure time, four fish for each feeding groups were sampled from each of their respective two tanks. Control groups (no HEA) were set up in parallel to the first (12 h) and the last sampling period (180 h) and were sampled in an identical way as for the exposure groups.

2.3. Sampling procedure

For sampling, fish (n=8) were removed from the tanks, anesthetized using an overdose of neutralized MS222 (pH 8.1, ethyl 3-aminobenzoate methane-sulfonic acid, 1 g/L, Acros Organics, Geel, Belgium), blotted dry and weighed. Subsequently, approximately 0.6-0.7 ml blood samples were withdrawn by caudal puncture into heparinized (2500 units/mL lithium heparin, Sigma, Munich, Germany) 1 mL syringes with 23-gauge needles. Blood was immediately centrifuged (for 1 min at the speed of 16,000 rpm at 4 ◦C), and aliquots of plasma were frozen in liquid nitrogen and stored at −80°C until later analysis. Fish were dissected on ice, gills, liver and muscle tissue was 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 RNA*later* (Qiagen, Hilden, Germany) and stored at 4°C for later molecular analysis.

2.4. Nitrogen excretion measurements

A parallel experiment was conducted to examine the ammonia and urea excretion rate. After 2 weeks of salinity acclimation, 16 fish (8 fed and 8 fasted) were transferred from their respective salinity-acclimated rearing tank to individual 8 L glass aquaria (water volume set to 5 L) with the salinity

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matching the acclimation salinity. The experimental aquaria were shielded with black plastic to minimize 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 ammonia exposure and left overnight to settle with continuous aeration. The experimental protocols consisted of exposing the fed and unfed fish with ammonia (1.17 ± 0.07 mM) in a similar way as done for 200 L tanks. To maintain the desired concentration of salinity and ammonia, 60–80% of the water was discarded every 2 days and replaced with fresh water containing the respective level of salinity and ammonia. Water pH was maintained constantly at 8.1. For the ammonia and urea excretion measurements, initial water samples (duplicate 2 mL) were taken at the beginning of the exposure followed by a final water sample collection after 12 h, 48 h, 84 h and 180 h. Similarly, this was carried for the control groups. Similar to the experiments conducted in 200L tanks, fed groups continued to receive feed throughout the exposure while unfed groups were kept starved.

Ammonia excretion rates J_{amm} (µmol/g/h) were calculated as:

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

where $[Amm]_i$ and $[Amm]_f$ are the initial and final concentrations of ammonia in the water (in µmoles/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}.

2.5. Analytical techniques and calculations

2.5.1. 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).

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2.5.2. Plasma metabolites

Ammonia levels in plasma were determined using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany). Plasma lactate concentrations were analyzed enzymatically using a commercially available kit (R-Biopharm AG, Darmstadt, Germany). Plasma [Na^+], [Cl^-] and [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).

2.5.3. Tissue energy store

Samples of liver and muscle were analyzed 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).

2.5.4. 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 HSI= (LM/BW) x100, where LM is referred as liver mass and BW is the body weight.

2.5.5. Enzymatic analyses

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

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2.6. Molecular analyses

RNA extraction from gill tissues, cDNA synthesis and quantification protocol for real-time PCR (qPCR) were carried out according to Sinha et al. (2015). The specific primers for Na^+/K^+-ATPase alpha subunit 1 (α-subunit1) and $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) is listed in Table 1.

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-1α and 18S rRNA) favoured elongation factor-1α (EF-1α) 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.

2.7. Statistical analysis

All data have been presented as mean values ± standard error (S.E.). 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 challenge, ammonia exposure and feeding status and their interactions were analyzed by three-way ANOVA. The data were analyzed 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. Principal component analysis (PCA) was performed by using OriginLab 9 software (OriginLab, Northampton, MA, U.S.A.). All measured parameters were subjected to PCA to investigate the overall effect of stressors on the physiological profile. The standardized scores of the first two components which explained the highest variation were used to make biplots.

No significant differences were found between any of the control values at 12 h and 180 h. Therefore, pooled controls for each experimental group are shown for clarity of the figures.

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3. Results

3.1. Ammonia and urea excretion rate

Comparison among control groups of seawater (32 ppt), brackish water (20, 10 ppt) and hyposaline water (2.5 ppt) acclimated fish shows that the ammonia excretion rate (J_{amm}) in fasted fish was inhibited significantly at 2.5 ppt compared to the respective seawater -acclimated fasted fish (Fig. 1). However, fed fish acclimated at lower salinities could retain J_{amm} analogous to the seawater acclimated fish.

HEA exposure to 32 ppt and 20 ppt acclimated fed and fasted fish induced an increase ($P < 0.05$) in J_{amm} (Fig. 1). In fed fish, such increment was seen even at 10 ppt, while in 10 ppt fasted fish J_{amm} was either reversed (at 48 h; $P < 0.001$) or inhibited (84 h-180 h; $P < 0.05$- 0.001). HEA exposure to both fed and fasted fish at hyposaline water (2.5 ppt) resulted in the inhibition of the excretion rate. The reduction of ammonia excretion was much more intense in fasted fish as they experienced a reversal from 12 h onwards. Overall, a significant interaction (Table 2) was observed between HEA, salinity challenge and feeding status on ammonia excretion rate.

For both feeding treatments, there was no effect ($P > 0.05$) of HEA exposure on urea excretion rate (J_{urea}) regardless of the salinity reduction (Fig. 2). However, an increasing trend ($P > 0.05$) was noted during 84 h- 180 h exposure for all salinity groups, marked by a numerical increment relative to the respective control.

3.2. Ammonia and urea accumulation

Under control conditions fasted fish at 2.5 ppt have a higher (57%, $P < 0.05$) plasma total ammonia (T_{amm}) level corresponding to the respective 32 ppt group (Fig. 3). The effects of HEA, nutritional status as well as salinity challenge were apparent in T_{amm} level. In 32 and 20 ppt acclimated fed fish, T_{amm} was significantly elevated during 84 h HEA exposure. This increase was followed by a subsequent recovery at 180 h to a value not significantly different from the control group. Unlike in fed fish, elevated T_{amm} persisted until the end of the exposure period (180 h) in 32 ppt and 20 ppt fasted fish. At 10 ppt, T_{amm} was elevated considerably ($P < 0.05$) in both feeding treatments from 48 h onwards compared to the

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respective control. Moreover, accumulation level in fasted fish at 84 h and 180 h were 44% ($P < 0.05$) and 60% ($P < 0.05$) higher relative to the fed fish. At 2.5 ppt, control fasted fish accumulated 46% higher ($P < 0.05$) ammonia than the respective control fed fish. At this salinity T_{amm} was elevated considerably ($P < 0.05$) in both feeding treatment following HEA exposure, but in fasted fish accumulations were much more intense (many fold increments, $P < 0.01$ or 0.001) in comparison to the respective fed groups. Overall effect of salinity ($P < 0.001$), HEA ($P < 0.001$) and food ($P < 0.001$) in individual as well as in combined form ($P < 0.001$) were noteworthy in plasma ammonia levels of fish (Table 2).

Fed and fasted fish displayed almost a similar pattern for urea concentration in plasma (Fig. 4). Significant increment in 32 ppt, 20 ppt and 10 ppt -acclimated fed fish was observed after 84 h -180 h of HEA exposure while in 10 ppt fasted fish the effect became prominent ($P < 0.05$) from 48 h onwards. At 2.5 ppt, significant increments for both feeding treatments were recorded following 48 h HEA exposure. No obvious differences ($P > 0.05$) between fed and fasted fish were reported at any of the sampling periods.

3.3. Osmolality

Salinity alone had a profound effect on plasma osmolality which was apparent only in the fasted fish (Fig. 5). Compared to 32 ppt control, osmolality was notably lower in fasted fish acclimated to10 ppt and 2.5 ppt with a 19% ($P < 0.01$) and 29% ($P < 0.001$) reduction respectively. Particularly at 2.5 ppt, osmolality was significantly lower in HEA-exposed fasted fish corresponding to their 32 ppt exposed groups. However, plasma osmolality for fed fish at different salinities remained unchanged throughout the experiment.

3.4. Muscle water content (MWC)

In fed fish MWC remained stable (Table 3). On the contrary, an effect of low salinity acclimation (at 2.5 ppt) was seen only in fasted fish, the MWC increased significantly by 21% from the control value.

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Similar to the plasma osmolality, no prominent effect of HEA was noted for MWC at different salinity regimes.

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

NKA activity in fish gills was affected by salinity, HEA and starvation (Fig. 6). Salinity reduction itself had a prominent effect in fed fish, the activity during 2.5 ppt acclimation increased by 80% ($P < 0.001$) relative to those reared at normal seawater (32 ppt). The exposure to HEA (84 h-180 h) at 32 ppt resulted in an increase in NKA activity in both fed and fasted fish. A significant rise was also noted for 20 ppt and 10 ppt fed and fasted fish following HEA exposure. In fed fish, the increment was recorded from 48 h onwards and persisted till last (180 h) exposure period; however such increment was postponed in fasted fish and became prominent only after 84 h. Exposure to HEA to 2.5 ppt had no effect ($P > 0.05$) on NKA activity for both feeding treatments, though an increasing pattern was observed for fed fish. The effect of feeding was seen only at the lowest salinity (2.5 ppt); NKA activity in fed fish gill augmented by 45% ($P < 0.01$), 62% ($P < 0.01$) and 66% ($P < 0.05$) respectively during control, 84 h and 180 h exposure compared to their fasted counterpart.

Branchial H^+-ATPase activity could not be detected in both feeding treatments when acclimated to 32 ppt, 20 ppt and 10 ppt (Fig. 7). However, the activity was perceptible when fish were reared at the lowest salinity (2.5 ppt). In contrast to their respective controls, both fed and fasted groups showed an increasing trend as ammonia exposure progressed in time. This effect was more prominent in fed exposed fish, relative fold increments were higher ($P > 0.05$) than in the fasted fish.

3.6. Gene expression of NKA

In general, NKA responses at gene level paralleled activity data quite well for both fed and fasted fish (Fig. 8). Similar to NKA activity, significant effects of salinity alone were seen on mRNA expression levels in gills of fed fish only. HEA induced an augmentation ($P < 0.05$- 0.01) in 32 ppt and 20 ppt acclimated fed and fasted fish from 84 h onwards. At 10 ppt such elevation ($P < 0.05$) in fed fish was

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seen from 48 h while it was delayed in fasted fish and became significant only after 84 h. In both feeding regimes no effect ($P > 0.05$) of HEA was observed in the hyposaline environment (2.5 ppt), while an increasing trend was noted for fed fish. The effect of nutritional status was apparent only at 2.5 ppt, a 1.44- fold ($P < 0.05$), 1.78- fold ($P < 0.05$) and 1.82- fold ($P < 0.05$) elevation was noted for fed fish over fasted fish respectively during control, 84 h and 180 h HEA exposure.

3.7. Gene expression of NKCC1

Branchial NKCC1 mRNA level in both fed and fasted fish reduced when subjected to lower salinities (Fig. 9). A 1.46 -fold reduction ($P < 0.05$) was observed for 10 ppt acclimated fasted individuals in comparison to the normal seawater (32 ppt) acclimated fasted fish. A more pronounced reduction (2.05-fold, $P < 0.001$) was evident when fasted fish were held at 2.5 ppt. Comparable reduction for fed fish was seen only at the lowest salinity (2.5 ppt) with a 1.54-fold ($P < 0.05$) reduction relative to the 32 ppt group.

On the contrary, HEA exposure tended to increase the transcript level of NKCC1 at all the salinities (Fig. 9). Expression levels augmented considerably ($P < 0.05$-0.001) in both fed and fasted fish compared to their respective controls during 84 h- 180 h HEA exposure.

3.8. Plasma ions

Following salinity acclimation to 10 ppt and 2.5 ppt, fasted fish displayed significant reduction in plasma [Na^+] relative to their 32 ppt control group (Table 3). However, HEA exposure at 32 ppt and 20 ppt resulted an increase in plasma [Na^+] in both fed and fasted fish from 84 h onwards. During10 ppt acclimation, significant increments in fed exposed fish compared to their control was seen from 48 h onwards while in fasted fish increments ($P < 0.01$) were noted only at the last exposure period. At 2.5 ppt, [Na^+] in fed fish following 84 h -180 h of HEA exposure prevailed the control by 25% ($P < 0.05$) and 30% ($P < 0.05$) respectively, and these elevations were also extensively higher ($P < 0.05$) than the corresponding fasted exposed group.

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No prominent effect of HEA or salinity challenge was evident for [Cl^-] in plasma of sea bass when reared at 32 ppt and 20 ppt (Table 3). The effect of osmotic stress became prominent only in fasted fish; an increment of 15% ($P < 0.05$) and 16% ($P < 0.05$) was seen in control groups of 10 ppt and 2.5 ppt respectively in comparison to the 32 ppt. The effect of HEA was noted for fasted fish at 10 ppt and 2.5 ppt which was marked by a significant elevation after 84 h -180 h exposure compared to the respective controls, 32 ppt exposed fasted fish and the corresponding 10 ppt and 2.5 ppt acclimated fed fish.

Plasma [K^+] did not change considerably under HEA or salinity reduction (Table 3). Moreover, no effect of nutritional status was observed.

3.9. Plasma lactate

Salinity reduction induced plasma lactate accumulation in feed deprived fish, which was significant at 10 ppt (29% increment, $P < 0.05$) and 2.5 ppt (45% increment, $P < 0.01$) compared to the normal seawater acclimated control group (Fig. 10). The effect of HEA in fasted fish was detected at 20 ppt, 10 ppt and 2.5 ppt whereas in fed fish similar augmentations were observed only at the lower salinities of 10 ppt and 2.5 ppt. Furthermore, a significant difference in plasma lactate was seen between fed and fasted fish during 84 h and 48 h of HEA exposure at 10 ppt and 2.5 ppt respectively; feed deprived fish accumulated higher plasma lactate compared to the fed fish.

3.10. Energy stores

In fasted fish, a distinct effect of salinity stress was noted on glycogen content in liver tissue; a significant reduction was observed in 10 ppt and 2.5 ppt control fish relative to 32 ppt (Table 4). A similar trend was noticeable for HSI (Table 4). Chronic HEA exposure (84 h-180 h) at 2.5 ppt reduced glycogen content in fed fish compared to the control as well as the parallel exposed group acclimated at normal seawater. For starved fish, effect of ammonia became prominent ($P < 0.05$) during 10 ppt and 2.5 ppt acclimation, a decline was noted from 84 h in the former while impact was seen a bit sooner (48 h

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onwards) in the latter salinity. A difference ($P < 0.05$) between the two feeding regimes was seen in all experimental salinities, hepatic tissue of fasted fish retained less glycogen store relative to the fed fish. However, for muscle tissue only a mild reduction ($P < 0.05$ or 0.01) in glycogen content was noted for both fed and starved fish under the combined effect of salinity and HEA (Table 4).

During acclimation to normal seawater and 20 ppt, protein content in liver tissue was reduced significantly in fasted fish following 84 h–180 h of HEA exposure in comparison to their respective controls and the fed fish counterparts (Table 4). The effect of HEA and salinity on protein content in muscle of both fed and starved fish showed more or less a similar pattern as for liver tissue.

Regardless of HEA exposure and salinity challenge, no significant alteration was seen for liver and muscle lipid store in fed and fasted fish (Table 4).

3.11. Principal component analysis (PCA)

The PCA biplot depicts a clear separation of experimental groups representing overall data, mainly along the first two components (PC1 and PC 2), together explaining 67% of data variability (Fig.11). These two components showed a clear separation indicating differential responses among fed and fasted fish which was classically prominent at hypo-saline water. The prevailing PC1 component (48% of the data variance) clustered J_{amm} ,T_{amm} , MWC, [Cl^-] and lactate content with starved fish under hyposaline stress on the one hand, and HSI and tissue energy stores with higher salinities for both fed and starved fish at the other hand. PC2 (19% of the data variance) clustered high salinities in both fed and starved fish with NKA NKCC and [Na^+], and low salinities in starved fish with J_{amm}.

4. Discussion

4.1. Effects on nitrogen metabolism

Ammonia is toxic and must be excreted to avoid internal hyperammonia events. In the present study, exposure to 1.18 mM ammonia at normal seawater (32 ppt) and at 20 ppt led to drastic increase in

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ammonia excretion rate (J_{amm}), suggesting that both fed and starved fish managed to keep excreting ammonia even against a concentration gradient (note that plasma T_{amm} levels remained < 0.4 mM). Despite the fact that J_{amm} increased considerably, plasma T_{amm} was slightly elevated (48 h-180 h) in fasted fish in contrast to plasma T_{amm} in the fed ones, so exposed fasted fish (at 32 ppt and 20 ppt) were not able to excrete ammonia as efficiently as fed fish. A prominent inhibition (and even reversal i.e., net ammonia uptake) of J_{amm} was seen for fasted fish when confronted with HEA at 10 ppt which resulted in a considerable difference in excretion rate between the fed and fasted exposed group. These responses were accompanied by substantial increments in plasma T_{amm} in fasted fish, indicating that the ability to transport ammonia against its concentration gradient was compromised. In fed fish the increase in plasma T_{amm} at 10 ppt were significantly lower (than fasted fish), implying that the effect of HEA is less detrimental at 10 ppt for fed fish. PC analysis also confirmed that feed deprivation is the prevailing factor influencing the ammonia homeostasis at reduced salinities when under HEA threat.

In addition, excretion rate was lowered in fasted fish in function of salinity alone, suggesting that ammonia metabolism can be adversely influenced under the synergistic effect of hypo-osmotic stress (2.5 ppt) and starvation. This divergent response among fed and fasted fish at 2.5 ppt acclimation and during HEA exposure at 10 ppt can be explained by the difference in metabolic status. It is well reported that under stressful situations food deprivation lowers the respiration rate in fish as an attempt to save energy stores and minimize energy investment (Scarabello et al., 1991; Wieser et al., 1992; Yang and Somero, 1993). In our previous similar studies in freshwater teleost (Diricx et al., 2013; Sinha et al., 2012a) we reported that high J_{amm} in ammonia exposed fed fish corresponded with an increase in oxygen consumption rate, indicating a higher metabolic rate. In general, ammonia is produced as a by-product of protein catabolism is excreted from fish gills either by diffusion of NH_3 or as the NH_4^+. Branchial transporters such as NKA and NKCC primarily associated with ion transport, are also involved in ammonia homeostasis since similarities in the hydration radius of K^+ and NH_4^+ allow substitution at transport sites (Alam and Frankel, 2006; Randall et al., 1999; Wilkie, 1997). NH_4^+ ions are then secreted in to the external milieu via the apical membrane in exchange of sodium through Na^+/H^+ (NH_4^+)

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exchanger (Weihrauch et al., 2009; Wilkie, 2002). It is tempting to speculate that the increased NKA activity in response to ammonia exposure at 32 ppt-10 ppt and a modest augmentation at hyposaline water (2.5 ppt) which occurred only in 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 (Patrick and Wood, 1999; Randall and Tsui, 2002; Wilkie, 1997). This might also help explaining in part the higher J_{amm} in fed fish (than fasted fish) during hypo-osmotic environment.

In addition, the majority of ammonia transport is thought to be through NH_3 diffusion either directly or facilitated by Rhesus (Rh) glycoproteins (Nakada et al., 2007; Nawata et al., 2007). Studies have shown that high external ammonia exposure in freshwater and marine teleosts triggers an upregulation of Rh mRNA expression in gills and thus enhances the J_{amm} (Braun et al., 2009; Hung et al., 2007; Nawata et al., 2007; Nawata and Wood, 2009; Tsui et al., 2009; Zimmer et al., 2010). Similarly, feeding has been shown to increase Rh transcript levels in juvenile trout gills under high water ammonia level (Zimmer et al., 2010) resulting in higher ammonia excretion rate in fed fish. These mechanisms appear to favor the findings of the current work, signifying that fed fish are better equipped to tolerate HEA compared to fasted fish. Though the sequence information (ESTs) for Rhcg2 for European sea bass is available (Boutet et al., 2006), molecular evidence of increased Rh protein expression in this fish species is at present still lacking.

Interestingly, analogous to fasted fish, ammonia excretion was also inhibited in fed fish when confronted with HEA at 2.5 ppt indicating that combined effect of ammonia and hypo-osmotic stress is detrimental even for fed fish. The reduction of J_{amm} in HEA- exposed fish at lowest salinity is probably not related to differences in branchial NH_4^+ permeability with seawater acclimated fish having an ion permeable gill epithelium facilitating ion excretion and possibly also promoting passive brachial NH_4^+ diffusion (Wilkie 1997, 2002) since plasma T_{amm} levels (0.22 - 0.37 mM) in HEA exposed seawater fish were considerably lower than the ambient water ammonia level (1.18 mM). It emphasizes the active role of transporters/exchangers, as ammonia exchange at the gills depends on the exchange of Na^+ for NH_4^+, it seems reasonable that the reduced availability of Na^+ in hyposaline water might compromise J_{amm}.

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Though ammonia is the main end-product of protein metabolism, a substantial proportion (10–30%) of nitrogenous wastes is also excreted as urea (Engin and Carter, 2001; Kajimura et al., 2004; Merino et al., 2007). Data presented in our study indicate that in contrast to J_{amm}, J_{urea} did not change significantly in response to salinity or HEA, but there were significant increments in plasma urea concentrations during HEA exposure. We speculate that in sea bass, the urea excretion mechanism was either very limited, or inhibited. However, the source of urea production in teleosts is still doubtful, potential pathways for the increased urea production in sea bass (e.g. uricolysis, arginolysis, ornithine–urea cycle) are worthy of future investigation.

4.2. Ion-regulatory adjustments

Branchial epithelium contains a number of transporters and co-ordinated mechanisms that are involved in maintenance of ion-regulation. NKA, present in the basolateral membrane of branchial cells is believed to provide the major source of energy driving Na^+ influx. It generates a low intracellular Na^+ gradient; thereby induce a 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 homologous to the human cystic fibrosis transmembrane conductance regulator (CFTR) (Hiroi and McCormick, 2012; Hwang et al., 2011). Lower salinities (2.5 ppt) activated the gene expression and activity of NKA significantly possibly as a compensatory mechanism to counteract ion loss, but only in the fed fish. The enhanced activity in fed fish aided to regulate their ion status and was reflected by the retention of Na^+ at the control level even in a hypo-osmotic environment, whilst fasted fish failed to do so. In euryhaline fish gill NKA activity has often been reported to be higher in freshwater than in the seawater-acclimated fish e.g. in European sea bass, milkfish (*Chanos chanos*), thicklip grey mullet (*Chelon labrosus*), pupfish (*Cyprinodon salinus*), mudsucker (*Gillichthys mirabilis*), Australian bass (*Maqcuaria novemaculata*), black seabream *(Mylio macrocephalus*) and killifish (*Fundulus heteroclitus*) (Doneen, 1981; Gallis et al., 1979; Katoh et al., 2002; Kelly et al., 1999; Langdon, 1987; Lasserre, 1971; Lin et al., 2003; Stuenkel and Hillyard, 1980).

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Following HEA exposure at different salinities (32 ppt-10 ppt) a rise in plasma [Na^+] in both feeding groups was accompanied by an increase in branchial NKA activity and activation of the transcript level of NKA-α1. NKA is not necessarily the only source driving Na^+ influx (Avella and Bornancin, 1989; Lin and Randall, 1995; Patrick and Wood, 1999; Randall and Tsui, 2002; Sinha et al., 2013; Wilkie, 1997), in freshwater fish H^+-ATPase present in the gill epithelium actively excretes H^+ across the membrane and generates a negative potential inside the apical membrane which drives Na^+ inwards via a sodium channel. However, its function is doubtful in marine fish. It was apparent from the present study that H^+-ATPase remains in the dormant state during seawater (32 ppt) and brackish water (20 ppt and 10 ppt) acclimation, while holding the fish in a hyposaline environment (2.5 ppt) necessitated the activation of H^+-ATPase to drive Na^+ uptake . Overall, it was apparent from our present study that as salinity decreases, sea bass rely primarily on the NKA empowering Na^+ uptake whereas contribution of H^+-ATPase for ion transportation is only important at very low salinities. Besides NKA and H^+-ATPase, NKCC is an important ion transport protein in marine teleosts (Lorin-Nebel et al., 2006; Marshall et al., 2002; Wilson et al., 2000) and is proposed to be involved in ion-secretion (Lorin-Nebel et al., 2006). A down-regulation of NKCC1 mRNA expression (in control groups) at lower salinities is probably an adaptive strategy of sea bass to rapidly restrict net ion loss, and corroborates with earlier findings in euryhaline fish species when transferred from seawater to freshwater (Hiroi and McCormick 2007; Katoh et al. 2008; Wilson et al. 2004; Wu et al. 2003). A prominent reduction in transcript level of this co-transporter at 10 ppt and 2.5 ppt among feed deprived control groups was concomitant with the increase in plasma [Cl^-]. It suggests that branchial secretion mechanisms are depressed to a larger extent by fasting resulting in hyperchloremia. Furthermore, it should be noted that the intense elevation in branchial NKA activity during ammonia exposure could also lead to a decline in the intracellular Na^+ concentration; this scenario predicts that the concomitant increase in NKCC1 transcript level during HEA exposure would be a countervailing response to maintain intracellular Na^+ level (Loong et al., 2012).

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In the present study, the gill co-transporter NKCC1 was investigated only at the mRNA level. Changes in gene expression do not always translate into comparable changes in protein function, although there was a fairly good correlation with the ion concentration. In future studies, investigation of NKCC1 at the translational level will be crucial.

4.3. Osmo-regulatory response

Plasma osmolality among fed fish in this study was essentially constant at experimental salinities and HEA exposure, suggesting osmoregulation homeostasis. Decline in the osmolality at lower salinities (10 ppt and 2.5 ppt) in feed deprived individuals might be a sign of osmoregulatory failure associated with a constraint in the adaptation process during hypo-osmotic challenge (Franklin et al., 1992; Nebel et al., 2005). Likewise, no impact of elevated water ammonia concentration and osmotic stress was reported in routine fed African catfish (*Clarias gariepinus*), rainbow trout, Atlantic salmon (*Salmo salar*) and turbot (*Scophthalmus maximus*) (Knoph and Olsen, 1994; Knoph and Thorud, 1996; Person-Le Ruyet et al., 2003; Schram et al., 2010; Vedel et al., 1998; Wilson and Taylor, 1992). During acclimation to an environment with varying osmolalities, the water content must be efficiently regulated in the fish's body to attain water balance. Muscle water content (MWC) is widely used as a physiological index to evaluate the water balance in fish; a change in muscle hydration is an important marker of osmoregulatory capacity and degree of euryhalinity (Freire et al., 2008; Tang et al., 2009). Our results revealed that acclimation to hyposaline water (2.5 ppt) resulted in considerable elevation of MWC in fasted fish compared to those at 32 ppt. This might have been a consequence of the enhanced osmotic water entrance, which also explains the fall in blood osmolality. Correspondingly, the MWC of the fed groups remained steady in all the tested salinities; indicating a better potential to sustain the water balance. Thus, feeding the sea bass definitely mitigates hypo-osmotic stress and facilitates osmoregulatory acclimation to adapt to the new environment.

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4.4. Energy metabolism trade-off

For the operation of various biochemical processes in osmoregulatory organs such as gills, glucose serves as the major metabolic fuelling source. Osmotic stress and elevated ammonia were seen to deplete glycogen stores, possibly to meet the energy demand required to fuel active NH_4^+ excretion operated by energy dependent ion-transporters. In fed fish glycogenesis and glycogenolysis almost remained in steady state (except at 84 h-180 h at 2.5 ppt) possibly because expenditures were efficiently replenishment by feed intake. The substantial depletion in glycogen store in unfed fish offers a justification for smaller hepatosomatic index, and might perhaps also have limited the activation of ion-transporters, explaining the disruption in ion-osmotic and ammonia homeostasis. It means that feeding facilitates the acclimation mechanisms during salinity stress and ameliorates ammonia handling via rendering energy available from food (Sucré et al., 2013). Our findings suggest that sea bass can change their energy substrate in conjunction with salinity, ammonia and feed deprivation. Fasting episodes at lower salinities (10 ppt-2.5 ppt) favored glycogen mobilization while at iso-osmotic salinities (32 ppt- 20 ppt) a preferential use of protein was noted. Similarly, red porgie (*Pagrus pagrus*) (Rueda et al., 1998) and European sea bass (McKenzie et al., 2014) at ambient salinity were reported to use tissue protein as a source of energy during 2- 3 weeks of starvation period. Fed fish also preferred glycogen during HEA exposure at the lowest experimental salinity, exemplified by the decline in glycogen content. These changes in the metabolic substrate towards glycogen instead of protein at lower salinities can be related to the lower nitrogen excretion in both feeding treatments.

Plasma lactate concentration has long been considered a useful indicator of aerobic limitations and anaerobic capabilities in stressful situations (Wendelaar Bonga, 1997). The reduction in glycogen content in fasted fish during lower experimental salinities (10 -2.5 ppt) corresponds with increment in plasma lactate. It highlights the onset of anaerobic metabolism and suggests the use of lactate as substrate for gluconeogenesis during osmotic adaptation, also reported by Polakof et al. (2006) in gilthead sea bream (*Sparus aurata*). In course of ammonia exposure in combination with salinity stress, the increase in anaerobic metabolism in both feeding treatments paralleled or followed by the

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mobilization of hepatic glycogen content. This strategy of the energy metabolism may be related to the fuel demand to maintain ion-regulation and for active NH_4^+ excretion when confronted with salinity reduction and ammonia threat simultaneously. The exact cause for anaerobic switching was not clear; it could be that sea bass exposed for long term ammonia and salinity stress were experiencing some form of hypoxia because these two environmental cues have been shown to disrupt gill epithelia in fish (Sinha et al., 2014; Swanson, 1998; Uliano et al., 2010; Wilkie, 1997). Regrettably, we did not measure oxygen consumption rate, so we do not know if the fish in these experimental situations experienced hypoxia.

5. Conclusion

We have determined the interactive effect of high environmental ammonia and nutritional status in European sea bass acclimated to experimental salinities from full strength seawater (32 ppt) to hypo-saline water (2.5 ppt). Analyzing the salinity effect alone, physiological and ion-osmoregulatory performance were strongly affected by feeding and fasting; fed fish revealed better hypo-osmoregulatory ability than the fasted fish. HEA itself (at 32 ppt) also had significant impact, and although the time course for the response was shortened in fed fish, the responses were nearly identical in both feeding regimes. Furthermore, the countervailing strategies against salinity stress were modulated when fish were confronted with HEA, and resulted in differential biological responses among fed and fasted fish. Firstly, in ammonia exposed fasted fish J_{amm} was significantly inhibited (even reversed) at 10 ppt acclimation, resulting in considerable accumulation of ammonia in plasma, whereas J_{amm} remained elevated in fed fish, thereby preventing the build-up of blood ammonia. Secondly, ion-regulation was severely affected in fasted fish by HEA exposure when reared at the brackish water (10 ppt) and typically at hyposaline water (2.5 ppt), evident by the reduction of $[Na^+]$ below the basal level and hyperchloremic events. In contrast, fed fish were able to maintain ionic homeostasis as they exhibited synchronized augmentations in NKA expression and activity. Thirdly, NKCC1 gene expression was down-regulated at 10 ppt (only in fasted fish) and 2.5 ppt (both in fed and fasted fish) which might

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enable the fish to rapidly decrease net ion secretion in hyposaline environment, while up-regulation following ammonia exposure probably aid to maintain intracellular Na^+ homeostasis. Interestingly, H^+-ATPase activity was expressed only at the lowest salinity (2.5 ppt) possibly assisting the fish for Na^+ uptake from the hyposaline media. Fourthly, at salinity range of 10 ppt and 2.5 ppt plasma osmolality in feed deprived HEA exposed fish was reduced and MWC was elevated while remained stable in fed fish. This may be a good indication of the improved osmo-regulatory capacity in fed fish in course of salinity and ammonia stress. Lastly, though only a modest alteration was recorded in fed fish, glycogen store in HEA exposed fasted fish (at 10 ppt- 2.5 ppt) subsided with a dramatic elevation in plasma lactate; signifying a shift towards anaerobic metabolism. In short, the patterns of physiological, metabolic, ion-osmoregulatory and gene-expression modulation observed in the present study indicates that feeding can mitigate the effects induced by salinity stress as well as ammonia exposure both alone and in combination. However, ammonia homeostasis was also disturbed when fed fish acclimated to lowest experimental salinity (2.5 ppt) were confronted with HEA. It indicates that the combined (synergistic) effect would probably be detrimental even for fed fish, despite their capacity to maintain ion-osmoregulation; suggesting a prioritization towards ion balance over ammonia handling under adverse environments.

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Figure Captions

Figure 1. Ammonia excretion rate (µmol/kg/h) in fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt -acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$).

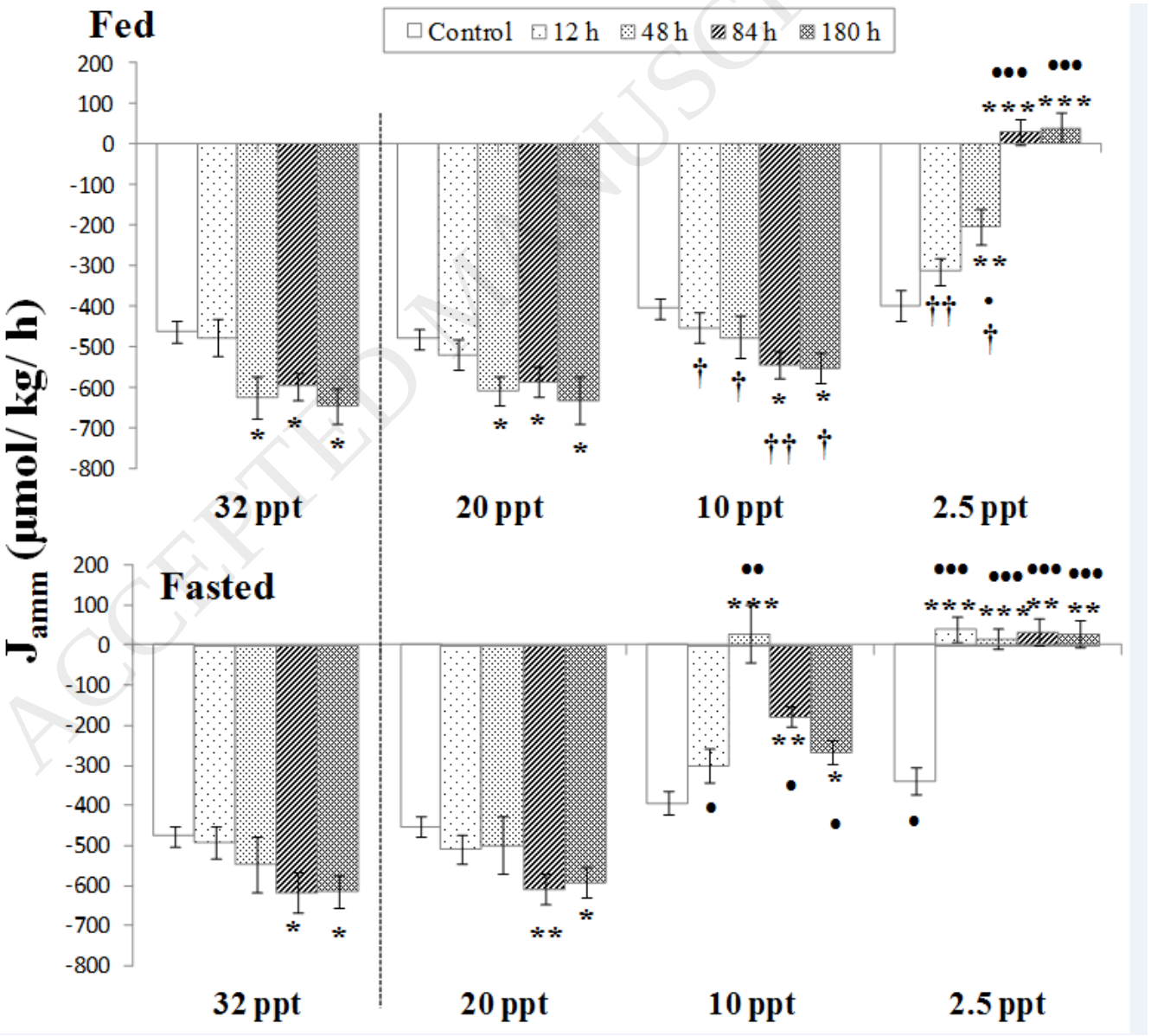

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Figure 2. Urea excretion rate (µmol/kg/h) in fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E.

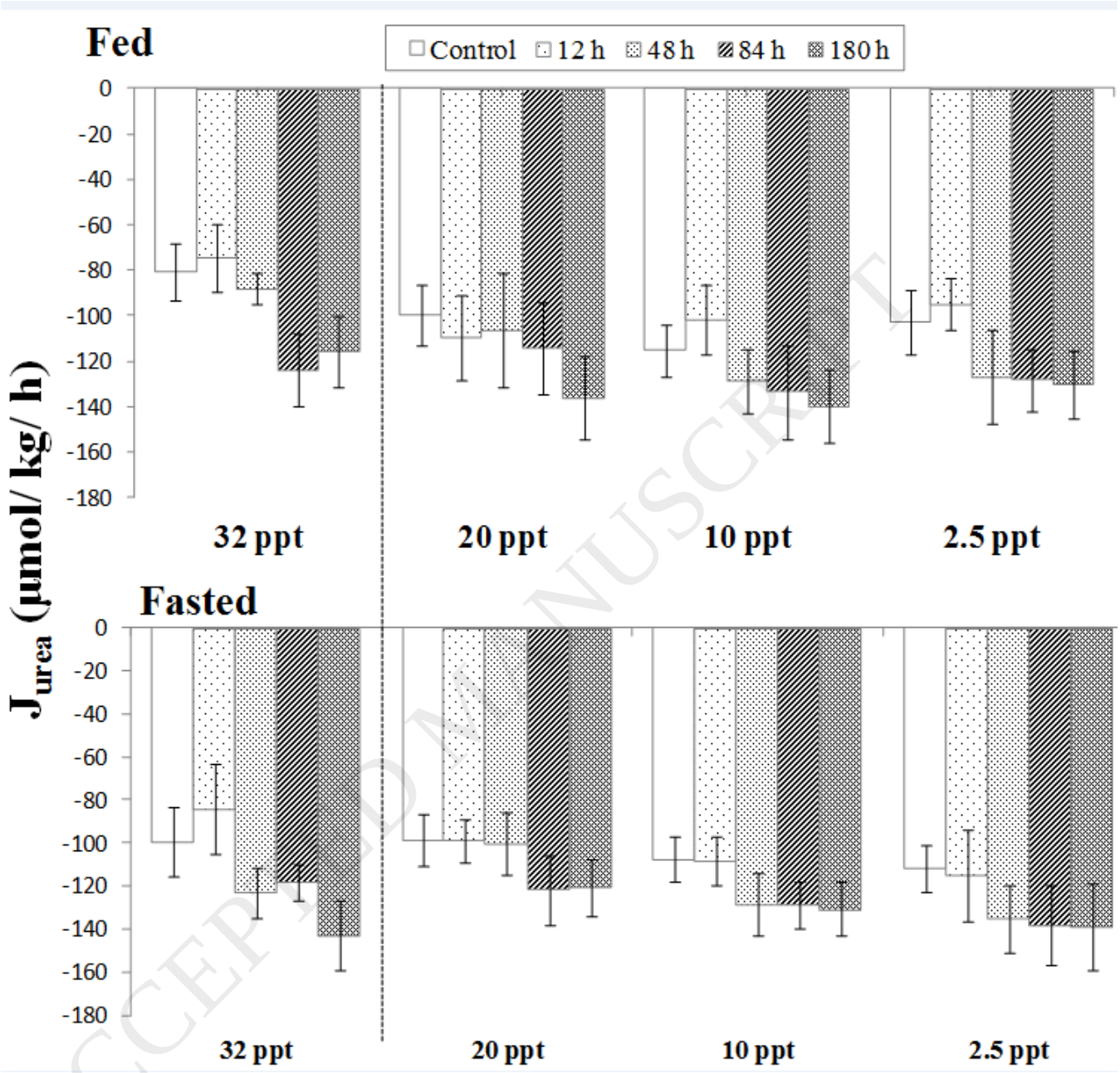

Figure 3. Total ammonia accumulation (mM) in plasma of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -

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2.5 ppt) and the 32 ppt -acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$).

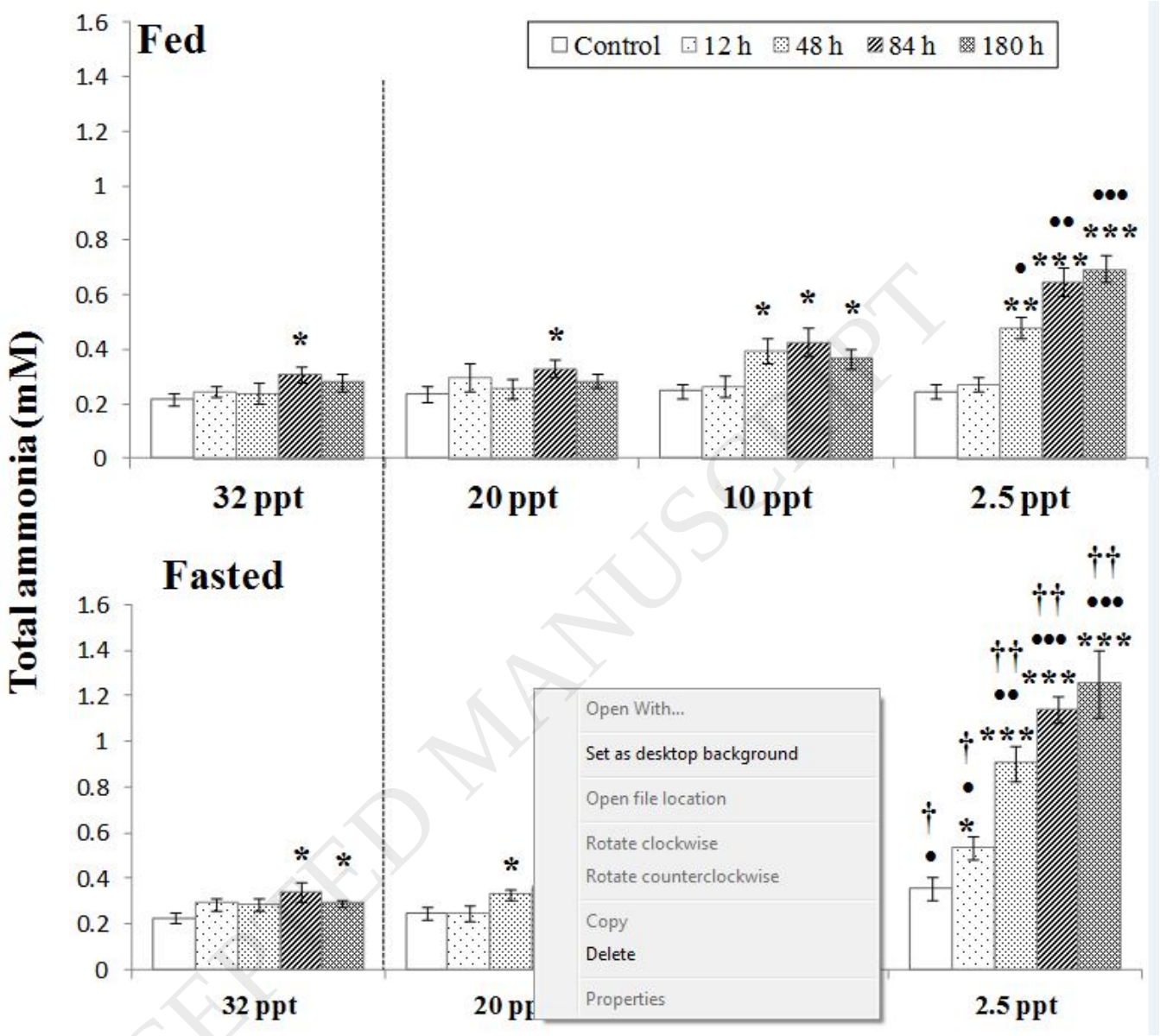

Figure 4. Urea accumulation (mM) in plasma of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$).

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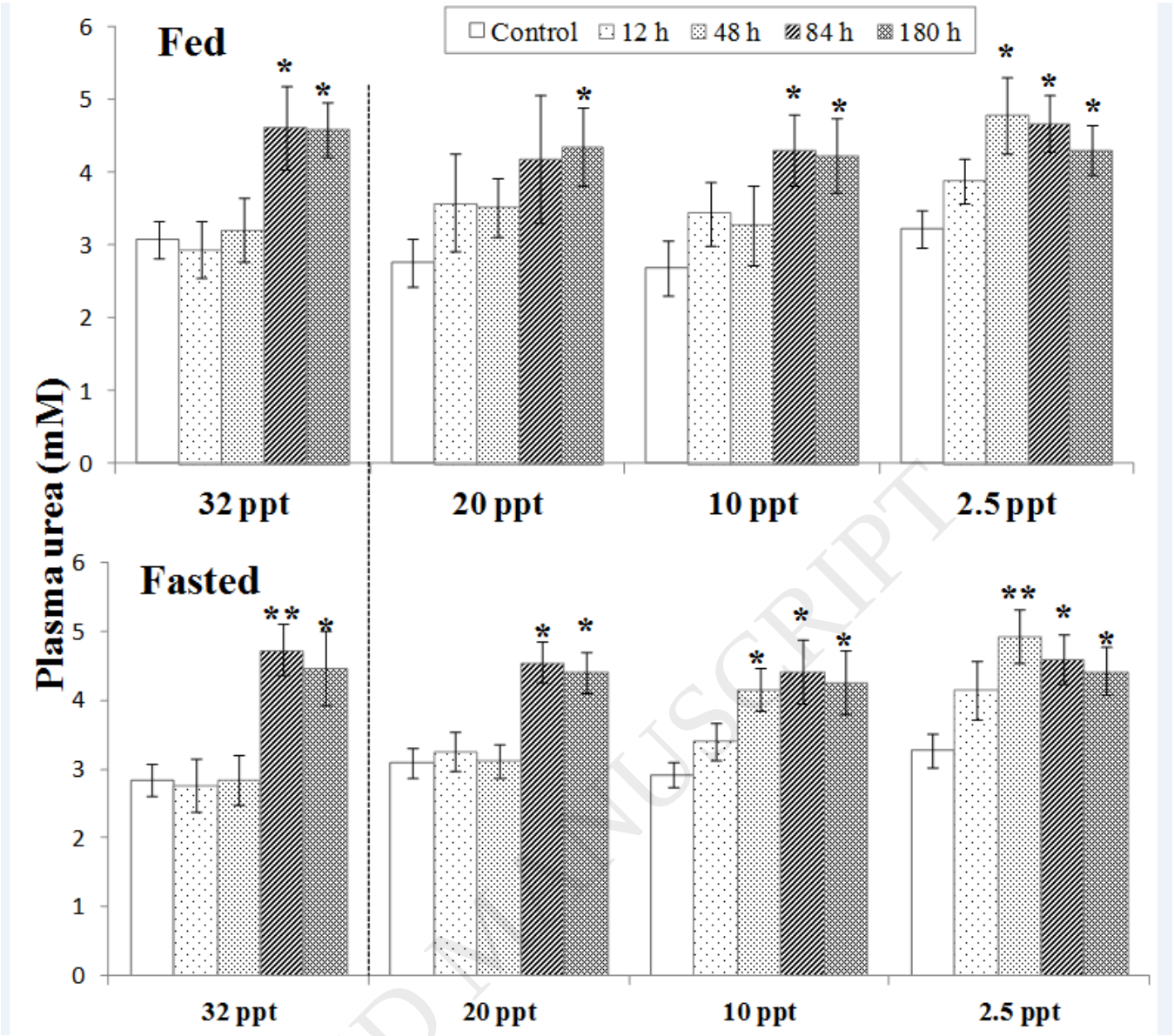

Figure 5. Osmolality (mOsm/kg) in plasma of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt -acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$).

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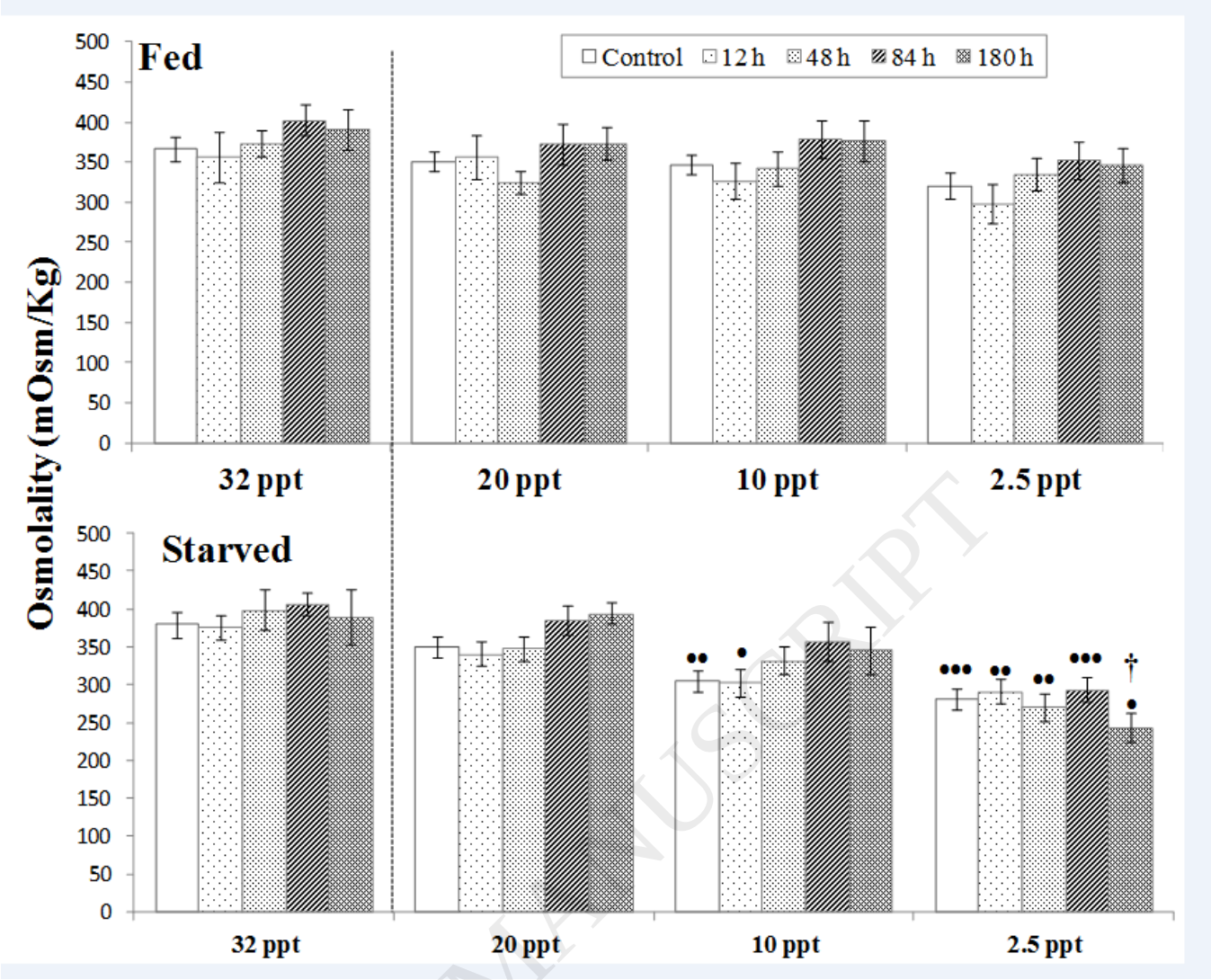

Figure 6. Na^+/K^+-ATPase activity in gills of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt - acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet\bullet}P < 0.001$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$).

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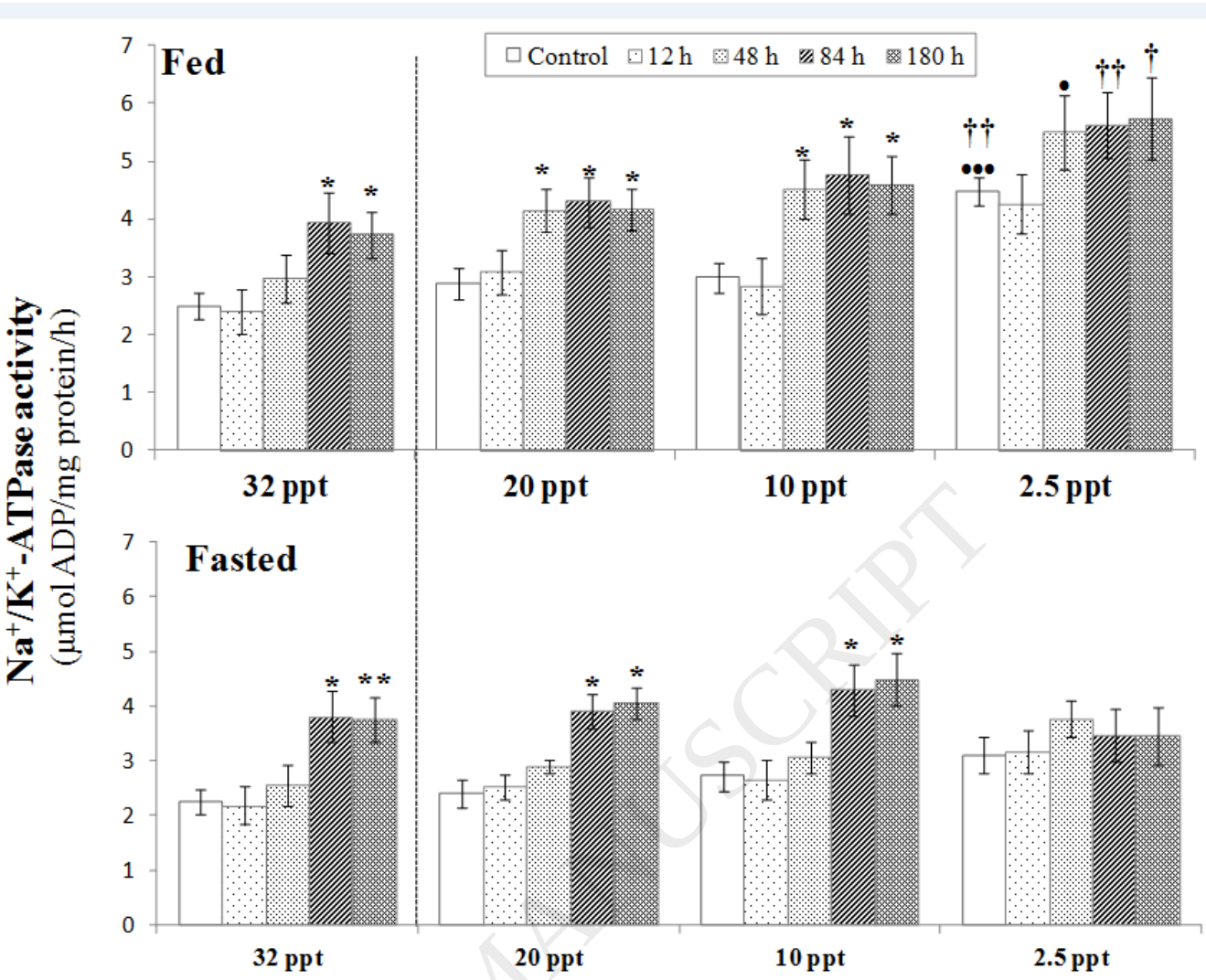

Figure 7. H^+-ATPase activity in gills of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E.

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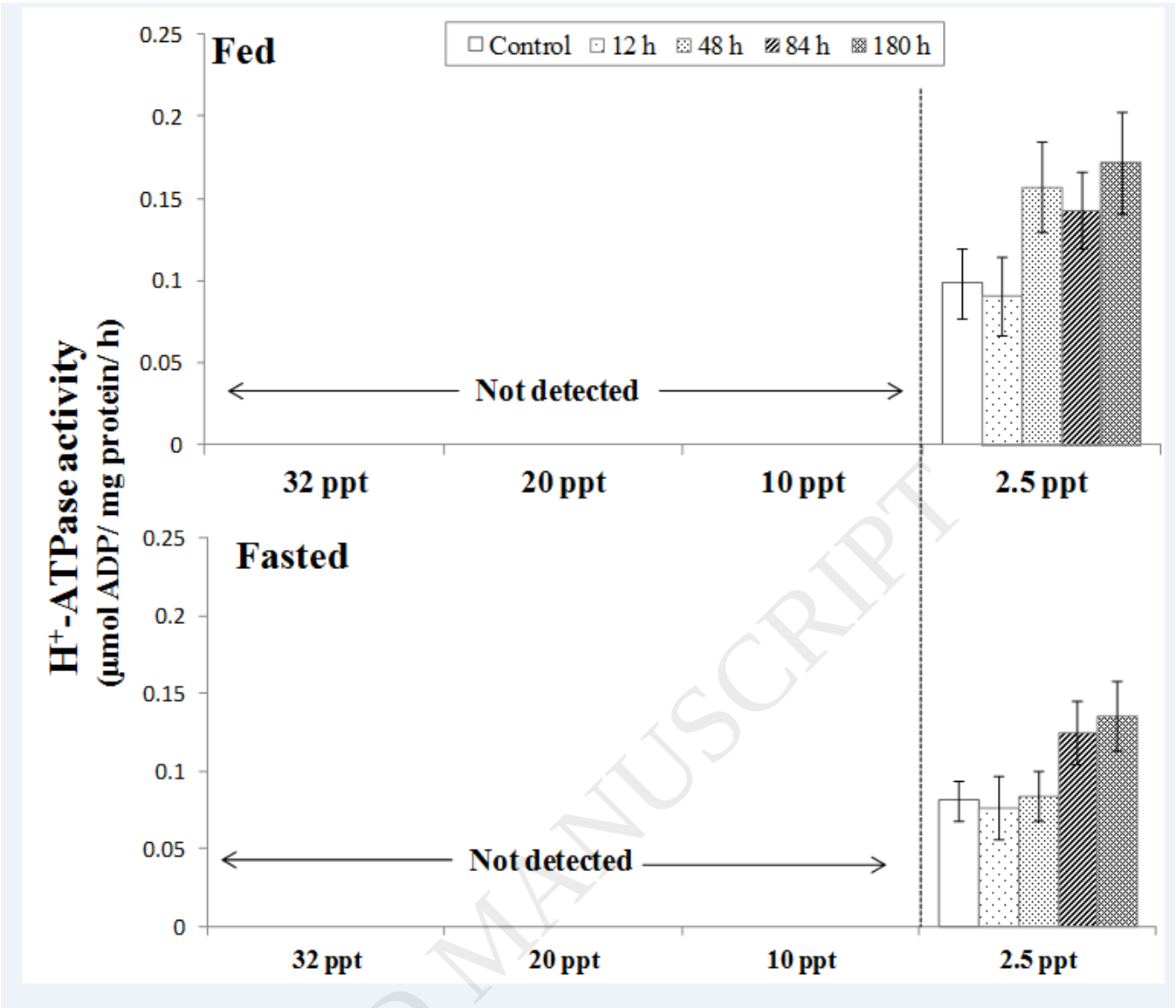

Figure 8. Na^+/K^+-ATPase expression in gills of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt -acclimated fish at the same sampling period ($^{\bullet\bullet}P < 0.01$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$).

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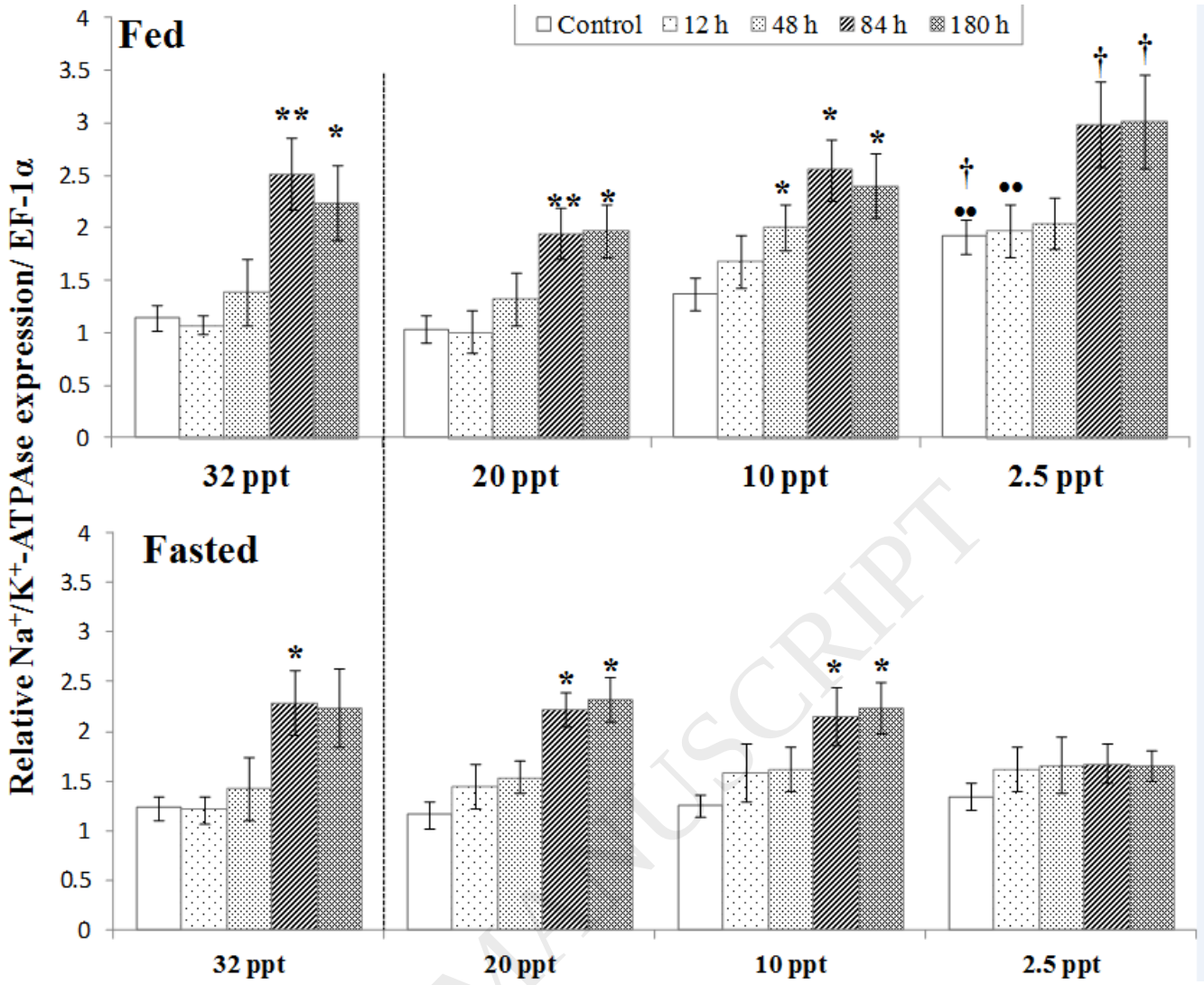

Figure 9. NKCC1 expression in gills of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt -acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet\bullet}P < 0.001$).

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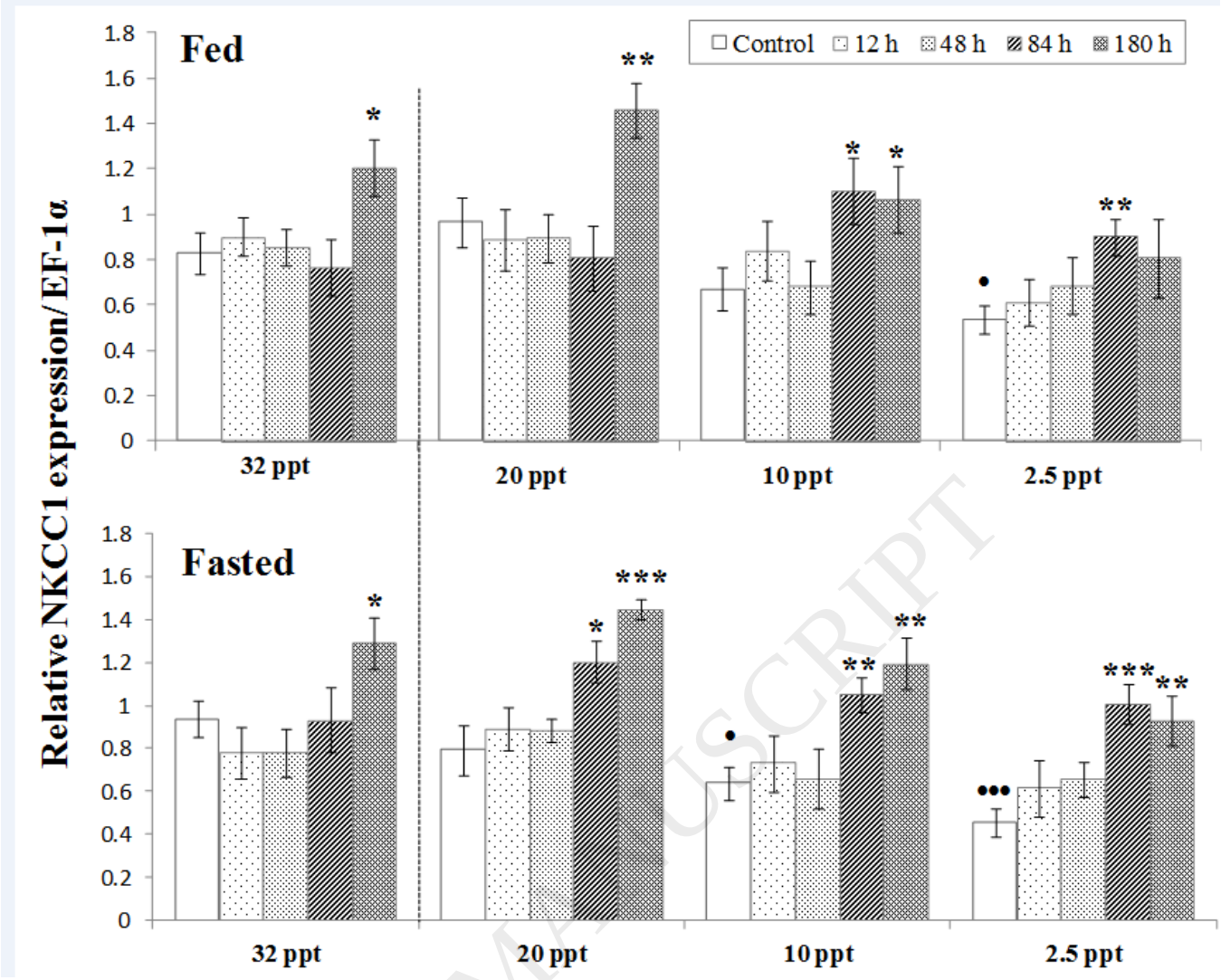

Figure 10. Lactate accumulation in plasma of fed and fasted fish during acclimation to different salinities and exposure to HEA. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt - acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$).

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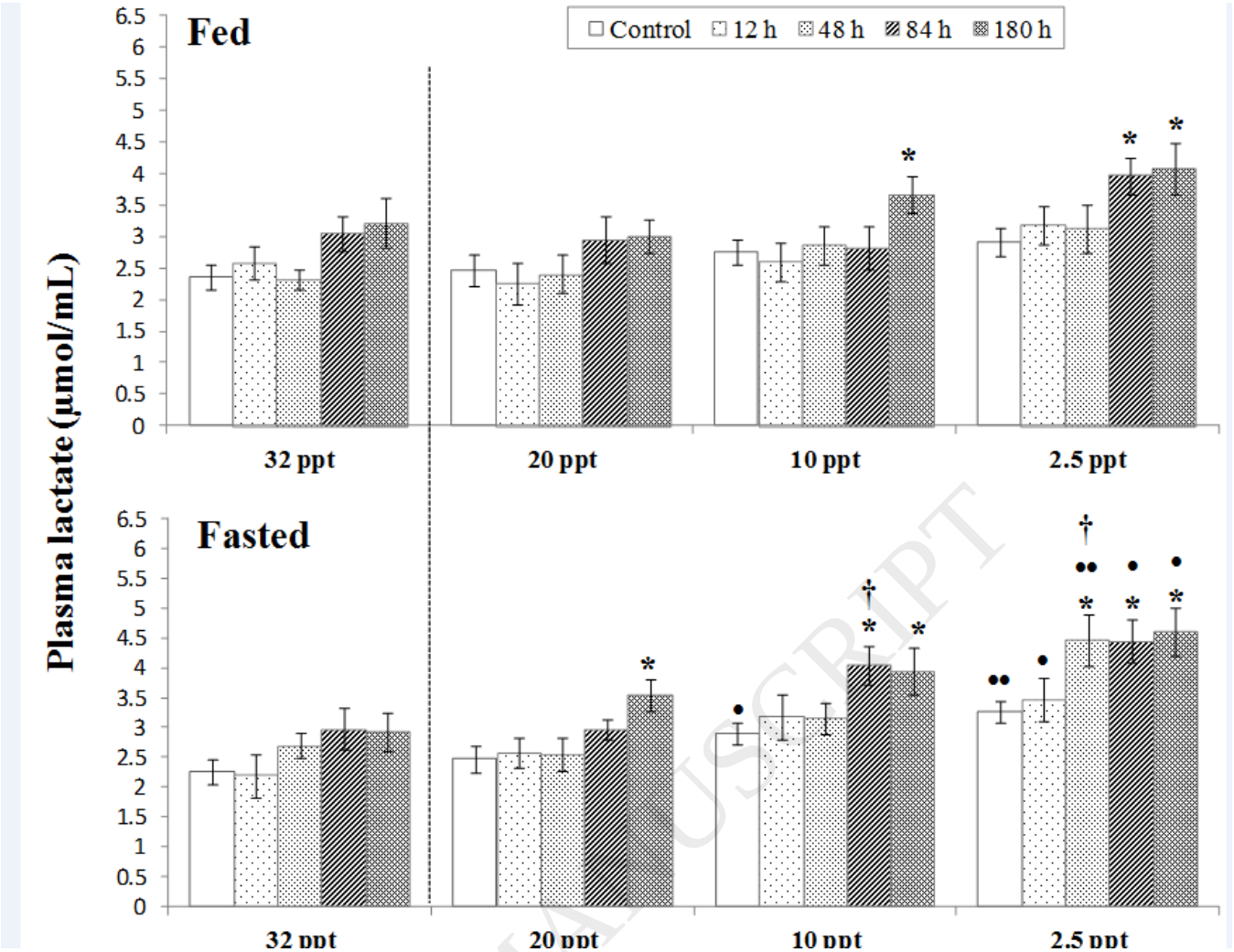

Figure 11. Principal Component Analysis (PCA) representing the contribution of biochemical parameters for fed (F) and starved (S) fish. The variable coordination is presented by the complementary cases analysis showing distribution of salinity acclimation groups (32, 20, 10, and 2.5 ppt) and HEA exposure (Control, 12 h, 48 h, 84 h, 180 h) in the (PC1 ×PC2) coordination plane.

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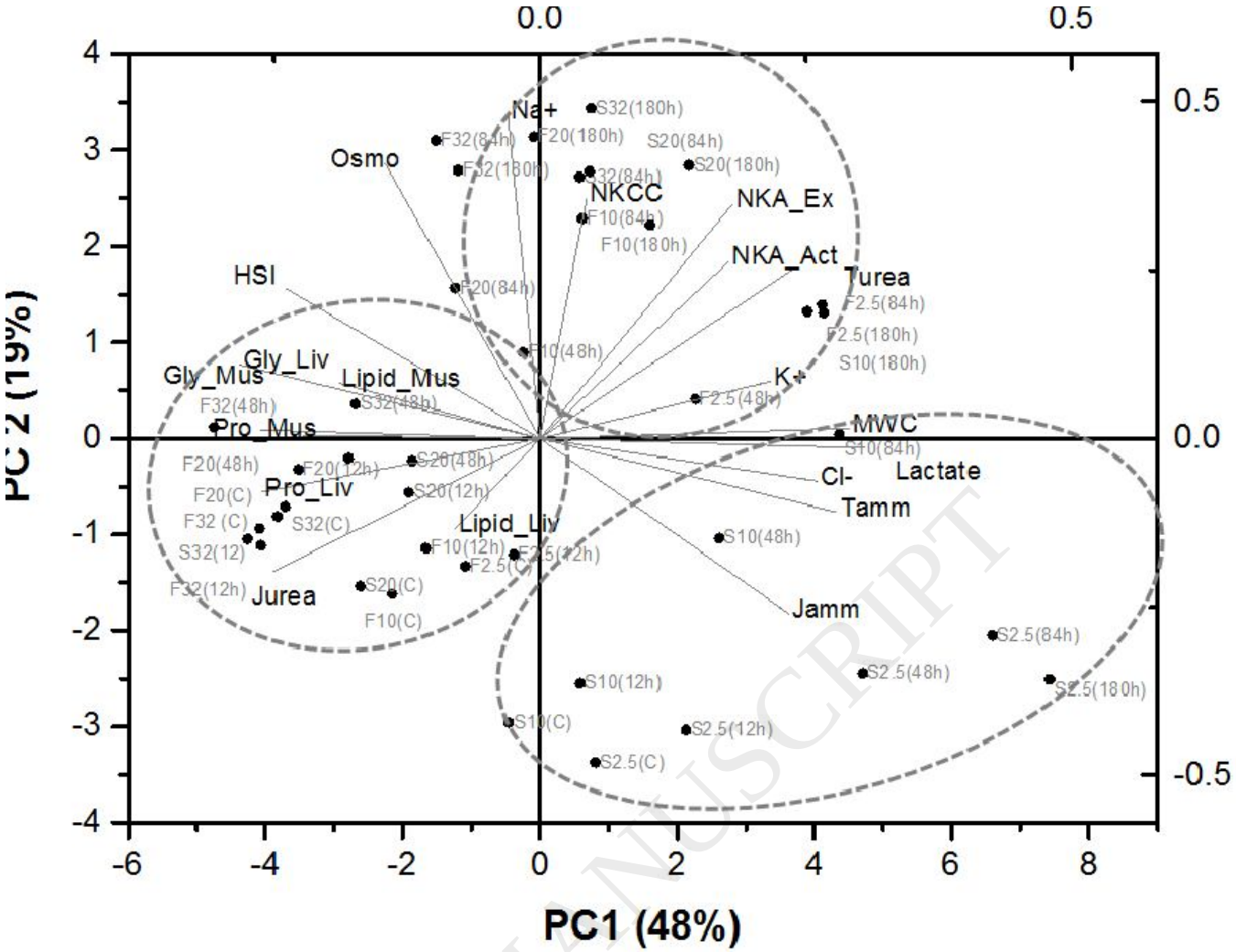

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Tables

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: CTGGAGTGGAAGAAGGTC 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: CGCTAGAGGTGAAATTCTTGGA Reverse: GATCAGATACCGTCGTAGTTCC	112%	Hakim et al., 2009	AM419038

The accession number refers to the registered sequence from GenBank.

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Table 2

The effects of salinity, ammonia exposure and feed and their interactions on metabolic, physiological and ionregulatory parameters in European sea bass.

Treatment	**Salinity**		**Ammonia**		**Feed**		**Salinity x Ammonia**		**Salinity x Feed**		**Ammonia x Feed**		**Salinity x Ammonia x Feed**	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
Non-invasive measurements														
J_{amm}	255.67	0.000	3.71	0.006	64.50	0.000	18.9	0.000	18.95	0.000	7.95	0.000	4.18	0.000
J_{urea}	2.89	0.036	6.38	0.000	0.999	0.318	0.253	0.995	1.24	0.295	0.043	0.996	0.146	1.000
Plasma metabolites														
Ammonia	181.87	0.000	78.13	0.000	140.62	0.000	20.55	0.000	41.39	0.000	9.92	0.000	2.93	0.001
Urea	4.34	0.005	21.35	0.000	0.110	0.740	1.35	0.191	0.363	0.780	0.058	0.994	0.250	0.995
Lactate	24.22	0.000	14.56	0.000	8.85	0.003	0.431	0.951	2.25	0.082	0.658	0.622	0.574	0.863
Osmolality	23.57	0.000	3.86	0.004	4.63	0.032	0.452	0.941	4.97	0.002	0.304	0.875	0.47	0.930
Ions														
Na^+	12.40	0.000	18.07	0.000	19.56	0.000	0.741	0.711	2.55	0.056	0.174	0.951	0.366	0.974
K^+	1.48	0.22	3.97	0.004	18.91	0.000	0.423	0.954	0.57	0.638	0.185	0.946	0.162	0.999
Cl^-	9.23	0.000	5.85	0.000	8.61	0.004	0.525	0.898	5.37	0.001	1.81	0.127	0.63	0.81
Branchial ion-transporters														
NKA activity	13.36	0.000	21.13	0.000	28.29	0.000	0.66	0.787	6.09	0.000	0.81	0.519	0.414	0.958
NKA expression	4.97	0.002	28.33	0.000	5.07	0.025	0.552	0.879	8.05	0.000	0.831	0.507	0.461	0.936
NKCC1	11.43	0.000	20.26	0.000	0.32	0.568	1.74	0.056	0.110	0.954	1.23	0.298	0.528	0.896

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expression

Liver metabolites													
Glycogen	24.75	0.000	7.73	0.000	66.80	0.000	0.724	0.728	2.19	0.088	0.74	0.563	0.988	0.460
Protein	10.59	0.000	7.29	0.000	17.07	0.000	0.599	0.843	2.35	0.073	0.412	0.800	0.459	0.937
Lipid	0.073	0.053	3.923	0.070	4.597	0.787	0.890	0.418	23.23	0.070	0.411	0.854	1.021	0.418
HSI	14.73	0.000	0.361	0.836	13.93	0.000	0.420	0.955	0.780	0.506	0.330	0.858	0.158	1.000
Muscle metabolites														
Glycogen	5.84	0.001	4.32	0.002	7.45	0.007	1.23	0.257	1.62	0.185	0.239	0.916	0.484	0.924
Protein	8.88	0.000	6.13	0.000	13.51	0.000	0.580	0.858	0.698	0.554	0.847	0.497	0.373	0.972
Lipid	1.08	0.301	4.267	0.000	10.612	0.001	0.428	0.883	4.964	0.0271	0.629	0.732	0.381	0.912
MWC	16.65	0.000	9.21	0.000	0.458	0.499	0.189	0.999	0.343	0.794	0.158	0.959	0.206	0.998

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Table 3

Ion concentrations (mmol/L) in plasma and MWC (%) in European sea bass under different treatments.

Treatments			Na^+	Cl^-	K^+	MWC
32 ppt	Control	Fed	174.4 ± 7.60	119.5 ± 3.96	2.53 ± 0.25	73.8 ± 3.19
		Fasted	160.4 ± 6.05	110.3 ± 4.41	2.46 ± 0.15	71.1 ± 3.39
	12 h	Fed	162.7± 11.60	121.0 ± 4.24	2.41 ± 0.37	69.8 ± 5.58
		Fasted	154.6 ± 6.80	112.3± 5.17	2.37 ± 0.24	69.6 ± 7.74
	48 h	Fed	173.5±10.71	112.8 ± 3.76	2.06 ± 0.38	77.6 ± 5.27
		Fasted	173.8 ± 12.77	109.5± 7.34	2.13 ± 0.33	78.7 ± 4.97
	84 h	Fed	207.6 ± 11.31*	118.8 ± 7.41	2.07 ± 0.29	78.0 ± 5.48
		Fasted	196.8 ± 11.52*	124.2± 6.01	2.05 ± 0.18	81.4 ± 5.50
	180 h	Fed	200.7 ± 7.70*	124.0 ± 7.51	2.01 ± 0.23	82.4 ± 5.97
		Fasted	197.3 ± 14.89*	118.0 ± 8.49	1.99 ± 0.29	79.1 ± 4.99
20 ppt	Control	Fed	159.8 ± 7.41	110.1 ± 4.55	2.69 ± 0.26	71.1 ± 2.98
		Fasted	150.1 ± 6.98	116.3± 3.53	2.77 ± 0.28	69.7 ± 2.98
	12 h	Fed	161.8 ± 9.32	116.5 ± 5.20	2.10 ± 0.18	69.7 ± 6.37
		Fasted	163.8 ± 8.46	114.3 ± 4.85	2.31 ± 0.25	76.4 ± 3.71
	48 h	Fed	166.8 ± 15.8	119.1 ± 3.63	2.39 ± 0.26	77.6 ± 4.70
		Fasted	157.7 ± 8.86	112.0 ± 3.02	2.23 ± 0.27	76.4 ± 3.94
	84 h	Fed	192.4 ± 12.3*	118.5 ± 5.40	2.09 ± 0.33	82.6 ± 5.21
		Fasted	188.2 ± 8.63*	124.6±4.76	2.14 ± 0.39	78.6 ± 3.72
	180 h	Fed	194.0 ± 11.70*	121.1 ± 5.58	2.17 ± 0.23	81.0 ± 5.43
		Fasted	180.8 ± 7.95*	123.3±3.29	2.25 ± 0.13	81.0 ± 3.81
10 ppt	Control	Fed	150.1 9.9	122.2 ±3.35	2.42 ± 0.20	81.9 ± 3.23
		Fasted	129.5 ± 11.00•	127.4± 5.40•	2.33 ± 0.23	79.6 ± 3.32
	12 h	Fed	148.4 ± 13.82	108.9 ± 7.56	2.69 ± 0.32	77.5 ± 4.16
		Fasted	127.2 ± 13.10	122.7± 7.69	2.45 ± 0.27	78.7 ± 6.23
	48 h	Fed	191.4 ± 11.4*	115.3± 8.19	2.35 ± 0.41	89.0 ± 5.13
		Fasted	161.4 ± 17.22	132.7± 6.67	1.90 ± 0.29	91.0 ± 7.00
	84 h	Fed	194.4 ± 13.40*	127.5 ± 8.26	2.08 ± 0.31	87.4 ± 6.42

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	180 h	**Fasted**	155.9 ± 15.21	146.3±5.60**•**	1.99 ± 0.25	93.6 ± 8.46
		Fed	192.4 ± 12.10*****	116.2 ± 6.94	2.12 ± 0.36	94.1 ± 6.88
		Fasted	188.5 ± 12.03******	144.3±4.21***•†**	1.98 ± 0.33	91.1 ± 6.08
2.5 ppt	**Control**	**Fed**	147.5 ± 11.10	124.5 ± 6.00	1.94 ± 0.29	82.5 ± 3.04
		Fasted	123.5 ± 10.15••	127.8 ±5.67•	1.89 ± 0.29	85.2 ± 3.71•
	12 h	**Fed**	146.4 ± 17.50	117.8 ± 7.60	1.96 ± 0.29	81.5 ± 6.98
		Fasted	119.5 ± 10.38•	121.9 ±2.33	1.91 ± 0.33	82.5 ± 3.74
	48 h	**Fed**	151.4 ± 14.20	124.6 ± 5.67	2.02 ± 0.42	90.0 ± 4.82
		Fasted	133.1 ± 12.65•	124.3 ± 4.40	1.94 ± 0.33	94.0 ± 5.45
	84 h	**Fed**	184.5±12.91***†**	128.6 ± 5.12	2.07 ± 0.29	92.3 ± 5.90
		Fasted	145.3 ± 10.39••	147.8 ± 6.25***•†**	1.92 ± 0.31	95.6 ± 5.49
	180 h	**Fed**	190.9 ± 12.71***†**	124.6 ± 6.21	1.96 ± 0.28	93.7 ± 5.36
		Fasted	139.6 ± 12.94•	153.0 ± 7.67***•†**	1.82 ± 0.27	103.3 ± 9.89

Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt -acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$).

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Table 4

Glycogen, protein and lipid content in liver (wet tissue) and muscle (wet tissue), and Hepatosomatic index (HSI) in European sea bass under different treatments.

Treatments			Glycogen (mg/g)		Protein (mg/g)		Lipid (mg/g)		HSI
			Liver	Muscle	Liver	Muscle	Liver	Muscle	
32 ppt	Control	Fed	162 ±7.2	8.2 ± 0.7	215 ± 8.8	239 ± 8.9	54.6 ± 6.8	24.7 ± 4.7	3.77 ± 0.18
		Fasted	153 ± 8.7	8.4 ± 0.7	203 ± 8.9	218 ± 5.9	48.7 ± 7.1	25.1 ± 5.9	3.62 ± 0.29
	12 h	Fed	157 ± 14.6	8.0 ± 0.8	224 ± 15.0	212 ± 16.4	41.4 ± 7.5	25.0 ± 3.4	4.01 ± 0.45
		Fasted	170 ± 15.0	9.3 ± 0.7	203 ± 8.9	200 ± 9.2	41.7 ±7.5	26.0 ± 4.3	3.77 ± 0.41
	48 h	Fed	184 ± 11.4	9.7 ± 1.2	220 ± 13.6	216 ± 13.6	49.8 ± 10.5	29.9 ± 3.5	3.88 ± 0.32
		Fasted	160 ± 16.5	9.9 ± 0.6	171 ± 11.9†	211 ± 20.5	42.3 ± 6.7	22.3 ± 3.2	3.66 ± 0.28
	84 h	Fed	179 ± 11.1	7.4 ± 0.7	200 ± 9.4	217 ± 21.7	47.0 ± 6.9	28.1 ± 2.2	4.13 ± 0.36
		Fasted	127 ± 8.6††	7.6 ± 1.0	162 ± 12.4*†	200 ± 16.3	41.4 ± 10.9	21.4 ± 4.3	3.76 ± 0.27
	180 h	Fed	173 ± 10.1	8.0 ± 0.9	193 ± 12.0	216 ± 8.3	44.7 ± 4.9	25.1 ± 3.7	3.57 ± 0.42
		Fasted	132 ± 8.9††	7.2 ± 0.9	166 ± 7.5*	180 ± 10.1**†	37.8 ± 12.8	23.9 ± 5.5	3.78 ± 0.39
20 ppt	Control	Fed	168 ± 7.2	8.2 ± 0.5	201 ± 8.3	210 ± 11.4	50.8 ± 4.4	29.3 ± 3.2	3.70 ± 0.33
		Fasted	151 ± 8.4	7.6 ± 0.5	185 ± 5.9	202 ± 7.8	53.6 ± 5.5	28.5 ± 6.8	3.34 ± 0.18
	12 h	Fed	172 ± 8.1	8.6 ± 0.7	212 ± 14.3	196 ± 26.6	46.3 ± 4.1	32.4 ± 3.0	3.56 ± 0.39
		Fasted	154 ± 10.7	6.6 ± 0.6	202 ± 8.9	171 ± 9.0	44.5 ± 8.4	32.6 ± 3.2	3.31 ± 0.18

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	48 h	**Fed**	173 ± 12.9	8.7 ± 1.1	206 ± 11.5	225 ± 23.4	48.4 ± 9.7	26.4 ± 3.6	3.40 ± 0.41
		Fasted	163 ± 9.4	7.7 ± 1.0	187 ± 6.2	192 ± 11.3	39.0 ± 8.0	30.7 ± 3.2	3.33 ± 0.23
	84 h	**Fed**	141 ± 9.0	8.7 ± 0.6	197 ± 7.9	194 ± 8.7	43.5 ± 14.8	26.9 ± 3.8	3.73 ± 0.25
		Fasted	135± 12.4	7.4 ± 0.5	158 ± 4.5**††	165 ± 4.4**†	38.6 ± 9.9	33.5 ± 5.9	3.23 ± 0.25
	180 h	**Fed**	152 ± 6.9	8.1 ± 0.7	190 ± 8.3	191 ± 8.2	40.2 ± 6.1	24.1 ± 2.8	3.67 ± 0.39
		Fasted	122 ± 6.6†	6.4 ± 0.4	162 ± 5.5*†	153 ± 6.3**†	42.1 ± 9.1	25.4 ± 5.7	3.14 ± 0.30
10 ppt	**Control**	**Fed**	167 ± 5.5	8.2 ± 0.5	190 ± 9.5	208 ± 6.5	54.1 ± 6.5	25.6 ± 3.5	3.28 ± 0.27
		Fasted	133 ± 6.2•††	7.4 ± 0.3	184 ± 8.7	192 ± 9.7	51.1 ± 6.6	25.7 ± 7.4	2.79 ± 0.19•
	12 h	**Fed**	157 ± 12.2	8.8 ± 0.7	174 ± 6.4	187 ± 10.2	48.9 ± 8.6	27.2 ± 2.5	2.93 ± 0.42
		Fasted	133 ± 7.4	7.1 ± 0.7	180 ± 6.0	179 ± 5.6	50.1 ± 6.7	25.7 ± 5.2	2.65 ± 0.32
	48 h	**Fed**	166 ± 12.1	7.4 ± 1.0	163 ± 10.5	187 ± 7.1	48.9 ± 11.8	26.6 ± 2.7	3.59 ± 0.31
		Fasted	136 ± 10.8	6.8 ± 0.5	156 ± 13.1	202 ± 5.2	38.7 ± 12.5	23.6 ± 3.2	2.85 ± 0.29
	84 h	**Fed**	148 ± 12.1	7.6 ± 0.5	181 ± 6.3	197 ± 7.7	56.0 ± 12.3	24.2 ± 3.3	3.23 ± 0.47
		Fasted	111 ± 5.5*†	6.9 ± 0.4	158 ± 13.4	178 ± 14.0	49.9 ± 4.6	26.0 ± 4.4	2.42 ± 0.22••
	180h	**Fed**	146 ± 12.6	6.7 ± 0.9	161 ± 16.6	201 ± 16.8	54.0 ± 8.9	25.9 ± 4.7	2.93 ± 0.44
		Fasted	109 ± 6.7*†	7.0 ± 0.9	163 ± 9.9	180 ± 14.3	45.3 ± 6.8	23.9 ± 3.6	2.83 ± 0.38
2.5 ppt	**Control**	**Fed**	155 ± 6.1	8.3 ± 0.6	186 ± 11.6	211 ± 10.0	51.1 ± 11.9	22.8 ± 3.2	3.35 ± 0.23
		Fasted	117 ± 7.6••†††	7.2 ± 0.4	175 ± 9.5	193 ± 10.8	49.6 ± 10.0	23.8 ± 5.9	2.90 ± 0.14•
	12 h	**Fed**	152 ± 11.7	8.5 ± 0.9	193 ± 17.7	186 ± 18.1	41.9 ± 13.3	21.0 ± 2.3	3.42 ± 0.10
		Fasted	111 ± 10.3•†	8.3 ± 0.5	174 ± 10.6	161 ± 6.8	45.4 ± 6.5	20.2 ± 3.8	2.88 ± 0.14
	48 h	**Fed**	150 ± 7.9	7.1 ± 0.5	168 ± 10.1	163 ± 14.1	39.4 ± 5.3	22.1 ± 3.3	3.46 ± 0.19

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84 h	**Fasted**	92 ± 8.4**••††	7.2 ± 0.8	166 ± 9.3	180 ± 13.1	47.0 ± 3.6	18.9 ± 4.9	2.93 ± 0.40
	Fed	124 ± 11.7**••	6.9 ± 0.4	167 ± 20.9	185 ± 11.6	48.0 ± 12.6	19.6 ± 3.5	3.12 ± 0.25
180h	**Fasted**	95 ± 5.1**•	4.7 ± 0.5**•†	172 ± 5.3	172 ± 14.8	47.6 ± 3.5	20.3 ± 5.2	2.34 ± 0.19••†
	Fed	125 ± 8.9**••	6.3 ± 0.4*	162 ± 16.7	183 ± 13.3	42.0 ± 11.5	20.9 ± 4.1	3.17 ± 0.32
	Fasted	96 ± 5.4**•	4.6 ± 0.4**•†	158 ± 16.1	166 ± 9.9	36.9 ± 8.9	21.0 ± 7.9	2.64 ±0.17•

Values are mean ± S.E. Asterisk (*) indicates a significant difference between the ammonia exposed fish and its respective control at each salinity ($^{*}P < 0.05$; $^{**}P < 0.01$), bullet (•) indicates a significant difference between experimental salinities (20 ppt -2.5 ppt) and the 32 ppt - acclimated fish at the same sampling period ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$), dagger (†) denotes the significant difference between fed fish and its respective fasted fish counterpart ($^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger}P < 0.001$).