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Cortisol affects metabolic and ionoregulatory responses to a different extent depending on feeding ration in common carp, *Cyprinus carpio*

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Abstract

This study investigated metabolic and ionoregulatory responses in common carp fed at 3.0% or 0.5% body weight (BW) which received either no implant, or a sham or cortisol implant (250 mg/kg BW) and were monitored throughout a 168 hour post-implant period (168h-PI). Immediately, cortisol implant induced an elevation in plasma cortisol, glucose and lactate levels. Plasma osmolality and ion levels remained largely unchanged, but cortisol increased gill and kidney Na^+/K^+ ATPase (NKA) and H^+ ATPase activities. Gill ionoregulatory activities were higher in high feeding fish, whereas kidney H^+ ATPase activity was greater in low feeding fish, especially at 12-24h PI. Cortisol induced liver protein mobilization and repartitioned liver and muscle glycogen. In high feeding fish, increased protein mobilization did not increase plasma ammonia and urea levels, reflecting an improved excretion efficiency with a significant Rhcg-1 upregulation. Cortisol induced upregulation of the kidney glucocorticoid receptors (GR1 and GR2) and mineralocorticoid receptor (MR) from 24h to 72h-PI in low feeding groups. GR2 and MR returned to control levels, but GR1 remained upregulated up to 168h-PI in low feeding fish. In high feeding fish, GR2 and MR were upregulated from 24h PI onwards. In the liver, all three receptors were upregulated in high feeding fish at 24h PI, whilst only GR2 and MR were upregulated in low feeding fish. In the gill, GR2 and MR were upregulated at 72h-PI and GR1 at 168h-PI in high feeding fish. Only GR2 was upregulated at 72h-PI in the gill of low feeding fish. We demonstrated that cortisol significantly induced *in situ* metabolic responses to compensate acute stress (12h-PI) followed by upregulation of ionoregulatory processes and corticosteroid receptors (24h-PI onward).

Keywords: Cyprinid, corticoid receptors, hydromineral balance, energy metabolism, Rhesus glycoprotein, stress

1. Introduction

Cortisol, the main corticosteroid is well known to play a pivotal role in the stress response and it is synthesized in the inter-renal cells of the head kidney. It regulates both glucocorticoid and mineralocorticoid responses which are ultimately determined by binding to corticoid receptors acting as ligand-inducible transcription factors; and achieving subsequent target gene expression (Bury et al., 2003; Bury and Sturm, 2007; Stolte et al., 2008a; Kiilerich et al., 2011). In principal, three corticoid receptors have been identified in teleosts, glucocorticoid receptor 1 (GR1); glucocorticoid receptor 2 (GR2) and a mineralocorticoid receptor (MR) (Bury et al., 2003; Bury and Sturm, 2007; Stolte et al., 2008a; 2009; Aruna et al., 2012a). These corticoid receptors respond with a different sensitivity to cortisol in transactivation and transexpression via high affinity and low capacity receptors in specific tissues or species (Bury and Sturm, 2007; Stolte et al., 2008a). This subsequently induces and regulates a wide range of physiological compensations and/or adaptations such as in intermediary metabolism, immune capacity, osmoregulation, growth and reproduction (Vijayan et al., 1997; Mommsen et al., 1999; De Boeck et al., 2001; Bury et al., 2003; Flik et al., 2006; Kiilerich et al., 2007; Stolte et al., 2008a; Chasiotis and Kelly, 2012; Teles et al., 2013).

Emerging *in vivo* and *in vitro* evidence shows that cortisol plays a significant role in ion homeostasis in freshwater fish (Babitha and Peter, 2010; Hwang et al., 2011; Chasiotis and Kelly, 2012; Kumai et al., 2012). Cortisol induces upregulation of Na^+/K^+ ATPase (NKA) activity which has been documented in several freshwater fish such as tilapia (Dang et al., 2000), channel catfish (Eckert et al., 2001); rainbow trout (Sloman et al., 2001); North African catfish (Babitha and Peter, 2010); goldfish (Chasiotis and Kelly, 2012) and zebrafish (Kumai et al., 2012). Recently, McCormick (2011) stated that cortisol induces increased

surface area in mitochondria rich cells and influences both branchial and renal function in freshwater teleosts, which subsequently affects the influx of Na^+ and Cl^-. The overall regulation behind this mechanism is believed to be modulated by the mineralocorticoid receptor (MR) which is responsible for maintaining ion homeostasis in teleosts (Gilmour, 2005; Bury and Sturm, 2007; Stolte et al., 2008a). Also our own investigations showed that cortisol not only upregulated gill NKA and H^+ ATPase activities in fish fed a high ration but also induced kidney NKA and H^+ ATPase activities in fish fed a low ration, indicating that feeding ration plays a modulating role as well (Liew, et al, unpublished).

In order to sustain homeostasis and survive under stress, extra energy expense is expected to fuel basal metabolism (Vijayan et al., 1997; Gregory and Wood, 1999; De Boeck et al., 2001). Subsequently, energy channelled towards somatic growth is limited, and a low growth and a 'high living cost' has been classified as a common secondary stress indication (Gregory and Wood, 1999). Hyperglycemia during hypercortisol is regulated by glucocorticoid receptors and provides easily accessible energy substrates (Bury et al., 2003). Furthermore, a similar increase in lactate level during cortisol elevation suggests the involvement of anaerobic metabolism (Pottinger, 1998). Health (1995) and De Boeck et al., (2001) stated that stress might increase aerobic or anaerobic metabolism or both. Our previous study showed that cortisol induced both aerobic and anaerobic metabolism simultaneously with greater plasma lactate elevation and ammonia excretion when swum actively (Liew et al., 2013a). This energy availability is also determined by the quantity of dietary food intake (Vijayan and Moon, 1992; Reddy et al., 1995).

Studies on the glucocorticoid receptors (GR1 and GR2), the mineralocorticoid receptor (MR) and the ion transporter (NKA and H^+ ATPase) mRNA expression in response to cortisol elevation have been performed by either challenging the fish with cortisol implants, salinity, husbandry stress or pollutants (Pottinger et al., 2000; Greenwood et al., 2003; Sturm

et al., 2005; Nawata et al., 2007; McCormick et al., 2008; Roy and Rai, 2009; Stolte et al., 2009; Kiilerich et al., 2011; Aruna et al., 2012a; Teles, 2013). In teleosts, cortisol is well known for its dual role: ion retention in freshwater fish and ion secretion in seawater fish (McCormick et al., 2008; Tipsmark and Madsen, 2009). However, the underlying molecular mechanisms and the signalling pathways still remain uncertain and may be species-specific, dose dependent and vary with condition (Kiilerich et al., 2011). Furthermore, there are controversial discussions about the involvement of GR in ionoregulation, and MR has recently been identified to be more sensitive to cortisol than GR in the process of controlling hydromineral balance (Greenwood et al., 2003; Sturm et al., 2004; Takahashi et al., 2006; Takahashi and Sakamoto, 2013). Feeding also plays a role in both energy metabolism and ionoregulation but information on the interaction between different feeding regimes and high levels of cortisol on corticoid and ionoregulatory mRNA expression is scarce. Therefore, the objective of the present study was to investigate the effect of cortisol on the expression of corticoid receptor, Rhesus glycoprotein and ion transporter (NKA and H^+ ATPase) genes in the gill, kidney and liver of common carp over a period of 168 hours-post cortisol implant (168h-PI) in common carp fed a high (3.0% BW) or low (0.5% BW) feeding regime. In our previous studies, we found that cortisol induced ionoregulatory responses in kidney rather than gill to enhance ion reabsorption in fish fed a low ration, whilst cortisol improved branchial ammonia excretion and clearance rate in fish fed a high ration (Liew et al., 2013b). Therefore in this study, we hypothesize that (i) hypercortisol will stimulate NKA and H^+ ATPase activities in the gill and improve ammonia excretion in high feeding fish and promote kidney ion reabsorption capacity in low feeding fish. Therefore, (ii) expression of gill Rhesus glycoproteins will be greater in high feeding fish to facilitate ammonia excretion and plasma ammonia level is maintained either below or at the same level as in low feeding fish. In order to address these hypotheses, plasma metabolites, plasma osmolality and ion

123 (Na^+, Cl^-, K^+) as well as NKA and H^+ ATPase activities in both gill and kidney were
124 measured. And lastly, we hypothesize that (iii) hypercortisol will induce GR1, GR2 and MR
125 expression and that this effect will be more prominent in liver and kidney of low feeding fish
126 to induce glycogenolysis and/or gluconeogenic actions. To address this hypothesis, liver and
127 muscle metabolites were also measured.
128
129 **2. Materials and Methods**
130
131 *2.1 Fish maintenance and experimental set-up*
132 A total of 200 common carp, *Cyprinus carpio* (23.9±1.2 g BW and 13.3±0.3 cm body
133 length (BL) (Mean±SEM)) purchased from commercial fish farm (Intratuin, Halsteren, The
134 Netherlands) were pre-acclimatized and distributed equally into two 500 L rectangular
135 fiberglass tanks (50×30×50 in.) in the aquarium facilities of the Systemic Physiological and
136 Ecotoxicological Research group at the University of Antwerp, Belgium with Antwerp City
137 tap water. Fish were acclimated for 8 weeks and fed at 1.5% BW per day with commercial
138 Koi pellet (Hikari Staple, Kyorin Food Ind. Ltd., Japan). Food nutrition values were ≥35%
139 protein, ≥3% lipid, 5% fibre, 13% ash, 1% phosphorus and 10% mixture of vitamins and
140 minerals values of 5.88 mg/g Na^+, 8.26 mg/g K^+, 2.62 mg/g Mg^{2+} and 7.80 mg/g Ca^{2+}. After
141 pre-acclimatization, per series (control, sham and cortisol implanted) and feeding regime
142 (low and high) 32 fish were randomly selected, weighed and distributed equally into four 50
143 L aquaria filled with Antwerp city tap water (pH 8.2±0.4) and acclimatized for another 6
144 weeks prior start the experiment. In this period, feeding regimes were adjusted to either a
145 high (3.0% BW) or low (0.5% BW) feeding ration. Pellets were divided over 2 equal portions
146 per day fed at 08:30 h and 18:30 h daily by slowly introducing pellets at the same corner of
147 each aquarium over a 20 min period. All pellets were consumed throughout the experiment.

Each aquarium was equipped with gentle aeration and a 6 L external filter consisting of fine filter wadding, activated carbon and ≈16 mm lava stone at a flow rate of 5 L/min. Aquaria were covered with a black plastic sheet to reduce visual disturbance. Room temperature was set at 17° C with a constant photoperiod of 12L:12D. About 80% of the water was renewed every 3 days with a semi-auto-siphoning procedure to avoid handling disturbance. Water temperature, NH_3/NH_4^+, NO_2^-, NO_3^-, pH were maintained at 16±0.8° C, <0.1 mg/L, <0.03 mg/L, <25 mg/L and pH 8.1±0.5 (7.8 - 8.4) respectively throughout the experiment.

2.2 Treatment groups and implantation

After acclimation, fish were subjected to control (no implant) and sham or cortisol implantation. Fish were anaesthetized individually in 0.1 g/L ehty-3-aminobenzoate methanesulfonic acid (MS222) (Acros Organics, Geel, Belgium) neutralized with 1N NaOH to the holding pH ≈8.1 (Antwerp City tap water pH) and weighed. Implantation was performed intraperitoneally with coconut oil at 10 µl/g of BW at 12h post feeding (Gregory and Wood, 1999; De Boeck et al., 2001a; McDonald and Wood, 2004; Liew et al., 2013b). Fish implanted with melted coconut oil only (≈27° C; Sigma Diagnostics) served as sham group. Fish implanted with a mixture of 25 mg cortisol (Hydrocortisone 21-hemisuccinate; Sigma Diagnostics) in 1 ml of melted coconut oil served as cortisol group. This dose was selected based on our preliminary test resulting in an overall dose of 250 mg cortisol per kg of fish. This dose significantly elevated cortisol levels and allowed recovery to control levels within 168 hours-post implantation (168h-PI). All implanted fish were briefly placed on ice for approximately 30s to facilitate solidification of the implant and placed in well oxygenated water to recover from unconsciousness. All groups (control, sham and cortisol) were monitored at 12h, 24h, 72h and 168h-PI intervals with eight fish in each group.

173 *2.3 Sampling*

174 Fish were carefully removed from the aquaria, anaesthetised (0.5 g/L, neutralized
175 MS222; Acros Organics, Geel, Belgium), quickly blotted dry and weighed followed by
176 sampling. Blood was drawn from the caudal vessels using a heparinised needle and syringe
177 within 1 min of sedation (2500 unit/ml lithium heparin, Sigma, Munich, Germany). The
178 blood was immediately centrifuged to 5,000 g at 4^{o} C for 1 min. Plasma was separated into
179 three 500 µl Eppendorf bullet tubes. Gills, kidney, liver and muscle tissues were wrapped in
180 aluminium foil. All samples were immediately frozen in liquid N_2 and stored at -80^{o} C for
181 further analysis.

182

183 *2.4 Biochemical analysis*

184 Plasma osmolality was measured on fresh plasma sample using The Advanced^{TM} Micro
185 Osmometer (Model 3300, Advanced Instruments, USA). Radioimmunoassay was performed
186 to measure plasma cortisol levels by using a commercial Cortisol ^{125}I RIA kit (ImmuChem^{TM},
187 MP Biomedicals LLC). Plasma glucose, lactate and ammonia were determined with
188 commercial Enzymatic Kits (R-Biopharm AG, Darmstadt, Germany). Plasma urea was
189 determined using the salicylate-hypochlorite assay (Rahmatullah and Boyde, 1980). Tissue
190 energy (protein, glycogen and lipid) content was determined in both liver and muscle tissues.
191 Protein was determined according to Bradford (1976) using a standard curve of bovine serum
192 albumin. Glycogen was measured using Anthron reagent with a glycogen standard curve
193 (Roe and Dailey, 1966). Total lipid was extracted by methanol-chloroform assay and
194 measured with a tripalmitin standard curve (Bligh and Dyer, 1959). All samples were read
195 with an Ultra microplate spectrophoto reader (ELx 808, Bio-Tek Instrument Inc., Winooski
196 VT, USA).

197

198 *2.5 Gill and kidney ATPase activity*

199 Na^+/K^+ ATPase (NKA) activity was measured according to the method described by
200 McCormick (1993) and H^+ ATPase activity was measured as described by Lin and Randall
201 (1993) modified by Nawata et al. (2007). Tissues were homogenized in ice cooled 4:1
202 SEI/SEID buffer solution (150 mM sucrose; 10 mM EDTA; 50 mM imidazole/SEI with 0.1%
203 sodium deoxycholate) and centrifuged (1 min at 5000 g, 4° C). Duplicate homogenates of 10
204 µl were pipetted into 96-well microplates in four series. For NKA measurement, a first series
205 of homogenate was mixed with 200 µl assay solution A (400U lactate dehydrogenase; 500U
206 pyruvate kinase; 2.8 mM phosphoenolpyruvate; 0.7 mM ATP; 0.22 mM NADH; 50 mM
207 imidazole) and a second series with 200 µl assay solution B (mixture assay A with 0.4 mM
208 ouabain). For H^+ ATPase measurement, a third series of homogenate was mixed with 200 µl
209 of assay solution C (mixture assay B with 500 mM NaN_3) and a fourth series with 200 µl of
210 assay solution D (mixture assay C with 100 mM NEM). The enzyme activities were
211 measured kinetically at 30 s intervals for 30 min at wavelength of 340 nm with a
212 spectrophotometer ($ELX808_{IU}$ Bio-Tek Instruments Inc. VT, USA). Calculation was
213 performed with a standard curve of ADP (Adenosine diphosphate). ATPase activities were
214 calculated by subtracting oxidation rate in the absence to the presence of ouabain for NKA
215 and NEM for H^+ ATPase. Unit of ATPase activity was expressed as µmol ADP/mg protein/h.
216

217 *2.6 RNA extraction and real time PCR*

218 Total RNA from gill, kidney and liver tissues were individually extracted by using
219 Trizol (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. DNAse
220 treatment was performed to avoid genomic DNA contamination by precipitating extracted
221 total RNA in ethanol, and the RNA pellets were dissolved in RNAase-free water. RNA

quantification was assessed by Nano-Drop spectrophotometry (NanoDrop Technologies, Montchanin, DE, USA) and samples were used if purity was >1.75 ratio (OD_{260}/OD_{280} nm absorption). RNA integrity was checked by denaturing gel electrophoresis through 1% agarose gel. An initial amount of 1 µg RNA was synthesized to First strand complementary DNA (cDNA) according to Revert Aid H minus First strand cDNA synthesis kit instructions (Thermo Fisher Scientific, Zellik, Belgium). cDNA was used as a template for quantitative PCR (qPCR) analysis for NKA, H^+ ATPase, Rhesus glycoproteins (Rhbg, Rhcg-A, Rhcg-B), GR1, GR2 and MR quantification. Amplification was performed in 20 µl mixture reaction volume containing 5 µl cDNA, 4 pmol each of forward and reverse primers, 0.8 µl calibration ROX dye (500× dilution) and 10 µl Platinum SYBR Green qPCR SuperMix-UDG (Brilliant® II SYBR® Green QPCR mastermix, Agilent Technologies, Santa Clara, CA) on specific PCR plates (BIOplastics BV, Landgraaf, The Netherlands). The PCR conditions were set as follows: amplification at 50° C (2 min) and 95° C (2 min); followed by 40 cycles initial denaturation at 95° C (15 s), annealing at 60° C (30 s) and extension at 75° C (30 s). Melt curve analyses of the reference genes and target genes were performed to obtain specific melting temperatures. Real-time quantitative PCR primer sets used for and NKA, H^+ ATPase, Rhbg, Rhcg-1 and Rhcg-2 were the same used by Sinha et al. (2013) (Table 1) and corticoid receptors (GR1, GR2 and MR) were the same as used by Stolte et al. (2008a) (Table 2).

A reference housekeeping gene (β-actin) was screened over 15 random samples and the threshold cycle (Ct) for β-actin exhibited only a small change across all time points and treatments. Therefore, β-actin was used for normalization as internal control to calculate relative mRNA expression by the standard curve method. Values were expressed as fold changes and Ct values were normalised to the housekeeping gene (β-actin) expression corrected for the efficiency of each primer set relative to control, and analysed according to

246 the Pfaffl method Relative Expression Software tool (REST©) (Pfaffl, 2001; Pfaffl et al.,
247 2002).
248
249 *2.7 Statistics analysis*
250 Normality was checked prior to analysis with the Shapiro-Wilk test. Homogeneity of
251 variance was verified using the Hartley test. Log-transformed data were applied if the
252 requirements for ANOVA were not fulfilled. Effect of feeding regime was assessed by
253 unpaired two-tailed student *t*-test. One way ANOVA was performed to examine significant
254 effects of implantation within and between treatments at different time points followed by
255 multiple comparison with the Tukey post-hoc test. Sample size for all biochemical data was *n*
256 = 8; while sample size for molecular assay was *n* = 8 for the control and sham implanted
257 groups and *n* = 4-5 for the cortisol implanted groups. The biochemical results showed that
258 both control and sham implanted groups were not significantly affected by the treatments.
259 Therefore, samples from these two groups were randomly selected and pooled serving as
260 controls for the molecular assay. Level of significance was set at $P<0.05$. All data were
261 expressed as mean values with standard error (Mean ± SEM).
262
263 **3. Results**
264
265 *3.1 Plasma metabolites*
266 Overall, plasma cortisol levels in both control and sham implanted groups exhibited no
267 significant difference throughout the entire experiment (Fig. 1). Cortisol implanted fish
268 showed a significant plasma cortisol elevation compared to control and sham fish. Plasma
269 cortisol levels peaked at 12h-PI and started to decrease at 24h-PI and returned to a level
270 similar to control and sham groups at 168h-PI in both feeding regimes (Fig. 1a and 1b).

A similar trend was observed for plasma glucose (Fig. 2a and 2c), although the glucose level still remained elevated as compared to control and sham implanted groups at 168h-PI in fish fed a low ration (Fig. 2a). Control fish that were fed a high ration exhibited relatively higher plasma glucose levels than the fish fed a low ration (Fig. 2a and c). An elevated plasma lactate level was only noticed during the first 12h-PI in cortisol implanted fish in both feeding regimes and recovered to control levels afterwards (Fig. 2b and 2d). This effect was significantly larger in fish fed a high ration (Fig. 2b).

Overall, low feeding fish accumulated higher plasma ammonia levels than high feeding fish, especially in the fish implanted with cortisol (Fig. 3a and 3c). The level of ammonia was significantly elevated at 24h-PI in high feeding fish (Fig. 3c), while this was seen at 24h and 72h-PI in low feeding fish after being implanted with cortisol (Fig. 3a). Control and sham implanted fish fed a high ration had higher plasma urea levels than cortisol implanted fish at 24h and 72h-PI (Fig. 3d), and in general urea levels were higher than in the fish fed a low ration (Fig. 3b). Cortisol implants only induced transient plasma urea elevation at 12h-PI in low feeding fish (Fig. 3b) and reduced plasma urea at 24-72h-PI in high feeding fish.

Osmolality levels remained stable in high feeding fish (Fig. 4e). A dip in plasma osmolality level compared to 12h-PInwas recorded in low feeding fish implanted with cortisol at 24h-PI but osmolality recovered afterwards (Fig. 4a). A feeding effect was observed at 168h-PI where high feeding fish implanted with cortisol exhibited higher osmolality levels than the low feeding fish (Fig. 4a and 4e). Overall, plasma ions (Na^+, Cl^- and K^+) remained at a relatively stable level (Fig. 4), although cortisol implanted fish seemed to display somewhat lower ion levels, but this was not significantly different. The only significant Na^+ loss was recorded in high feeding cortisol implanted fish at 24h-PI (Fig. 4f). Additionally, there was a feeding effect for Na^+ in control fish at 24 and 72h-PI and in cortisol implanted fish at 168h-PI with higher Na^+ levels in fish fed a high ration (Fig. 4f).

296

297 *3.2 Gill and kidney ATPase activities*

298 NKA activity increased significantly after cortisol implantation in the gill and kidney of
299 both feeding regimes (Fig. 5). In the gill, cortisol induced a similar trend with greater NKA
300 activity observed at 24h-PI in both feeding regimes fish (Fig. 5a and 5c). Although fish fed a
301 high ration had greater gill NKA activities overall, a significant increase compared to low
302 feeding fish was only recorded at 24h-PI and 72h-PI in sham and cortisol implanted fish; and
303 at 72h in control fish (Fig. 5a and c). In the kidney, a significant increase in the NKA activity
304 was only recorded at 24h-PI in fish fed a high ration (Fig. 5d). In fish fed a low ration, this
305 significant increase occurred at 12h-PI and 24h-PI (Fig. 5b).

306 Gill H^+ ATPase activity remained unaltered after cortisol treatement, although there
307 was a trend towards increased H^+ ATPase activity in cortisol implanted fish (Fig. 6a and 6c).
308 A feeding effect was observed at 12h-PI with greater activity in sham and cortisol implanted
309 fish, and at 24h-PI in control fish fed a high ration (Fig. 6c). In the kidney of fish fed a high
310 ration, kidney H^+ ATPase activity remained stable (Fig. 6b). Compared to the gills, an
311 opposite trend was observed in the kidney with greater activity in fish fed a low ration.
312 Additionally, cortisol implantation significantly upregulated kidney H^+ ATPase activity at the
313 first 24h-PI in fish fed a low ration (Fig. 6b).

314

315 *3.3 Tissue metabolites*

316 Cortisol induced liver protein mobilization in both feeding groups and mobilization was
317 initiated at 12h-PI (Table 3). In fish fed a high ration, this mobilization was observed for the
318 first 24h-PI only. In low feeding fish, cortisol significantly mobilized liver protein up to 72h-
319 PI. Surprisingly, a remarkable muscle protein mobilization was observed in high feeding fish
320 implanted with cortisol at 24h-PI (Table 4), whilst muscle protein was maintained stable in

low feeding fish. High feeding resulted in higher glycogen reserves in both liver and muscle. Cortisol induced liver glycogen deposition in both feeding groups (Table 3). In fish fed a high ration, the effect of cortisol induced glycogen deposition was only observed at 24h-PI. However, the effect of cortisol was more prominent in low feeding fish with a significant glycogen deposition observed from 24h-PI onward. Muscle glycogen deposition was remarkable in high feeding fish at 72h-PI (Table 4), whereas muscle glycogen remained at a relatively stable level in low feeding fish. Overall, high feeding fish implanted with cortisol had higher muscle glycogen content than low feeding fish (Fig. 8b). Both liver and muscle lipid contents remained relatively stable in both feeding regimes (Table 3 and 4). A liver lipid mobilization was only observed in high feeding fish implanted with cortisol at 168h-PI as compared to sham implanted fish.

3.4 Gene expression

The expression of NKA and H^+ ATPase mRNA in the gill and kidney is shown in Fig. 7. In the gill, neither feeding nor cortisol implants have an effect on NKA and H^+ ATPase expression, although an increasing trend was observed during the first 72h-PI with cortisol implants (Fig. 7a and 7c). In the kidney, cortisol significantly upregulated NKA expression in fish fed a low ration from 72h-PI onward (Fig. 7b), while no effect was observed in fish fed a high ration (Fig. 7d). Similarly, no significant effect was observed in H^+ ATPase expression over time, although an increasing trend was noticed (Fig. 7b and 7d). H^+ ATPase expression tended to be higher in kidneys in high feeding fish, a significant feeding effect was seen at 24h-PI.

Overall, gill Rhesus glycoprotein (Rhbg, Rhcg-1 and Rhcg-2) expression stable in low feeding fish (Fig. 8a). In high feeding fish, an increasing trend of Rhbg, Rhcg-1 and Rhcg-2

345 expression was seen after cortisol implantation, but only Rhcg-2 expression was significantly
346 increased from 72h-PI onward (Fig. 8b).

347 The corticoid receptors (GR1, GR2 and MR) expression pattern is summarized in Fig.
348 9. In the gill, GR1, GR2 and MR receptors were expressed in a similar way in both feeding
349 regimes (Fig. 9a and 9d), but were influenced by cortisol, especially in the high feeding fish
350 (Fig. 9b). In the low feeding fish, the gill GR2 was significantly upregulated by cortisol
351 implantation at 72h-PI and returned to control level at 168h-PI (Fig. 9a). In the high feeding
352 fish, both gill GR2 and MR were upregulated at 72h-PI, while at 168h-PI only GR1 was
353 upregulated (Fig. 9b).

354 In the kidney, GR1, GR2 and MR expression levels were more prominent in cortisol
355 implanted fish fed a low ration compared to those fed a high ration (Fig. 9b and 9e). In the
356 high feeding fish, GR2 and MR were upregulated from 24h-PI onward (Fig. 9e). In the low
357 feeding fish, kidney GR1 was significantly upregulated from 24h-PI onward. GR2 was
358 significantly downregulated at 12h-PI and was significant upregulated at 24h-PI and 72h-PI
359 (Fig. 9b). Similarly, MR expression levels were significantly upregulated at 24h-PI and 72h-
360 PI (Fig. 9b). A feeding effect was observed in the kidney GR2 and MR at 24h-PI with higher
361 expression rates in low feeding fish (Fig. 9b), while the expression levels were greater in high
362 feeding fish at 168h-PI (Fig. 9e). Liver corticoid receptors showed a peak upregulation at
363 24h-PI (Fig. 9c and 9f). A significant upregulation of liver GR1, GR2 and MR was detected
364 at 24h-PI in high feeding fish (Fig. 9f), whereas in the low feeding fish, GR2 and MR were
365 significantly upregulated at 24h-PI (Fig. 9c).

366

367

4. Discussion

4.1 Metabolic and ionoregulatory responses

Food limitation had no effect on the plasma cortisol changes that were induced by the cortisol implants. This result is similar with our previous study showing that plasma cortisol elevation was primarily caused by the cortisol implants without being affected by either food deprivation or exercise in common carp (Liew et al., 2013b). Also other studies showed that food deprivation had no effect on plasma cortisol concentration in other species (Farbridge and Leatherland, 1992; Vijayan and Moon, 1992; 1994; Jørgensen et al., 1999). Cortisol level started to decrease as soon as 24h-PI and recovered to control level at 168h-PI likely associated with active cortisol catabolism and cortisol clearance. Mommsen et al. (1999) and Teles et al. (2013) described that increased cortisol catabolism and negative feedback systems allowed teleosts to acclimatize to chronic cortisol elevation.

Our results show that fish fed a high ration had higher glucose level than fish fed a low ration during hypercortisol and that the level of glucose recovered parallel to the cortisol recovery. In fact, hyperglycemia was reported previously in carp responding to cortisol elevation (Pottinger, 1998; Pottinger et al., 1999; 2000; 2010; Ruane et al., 2001; 2002; Liew et al., 2013b). In conjunction with increased plasma ammonia and urea levels, liver protein was mobilized indicating that protein was used for gluconeogenesis. Cortisol induced protein catabolism has been found before in salmonid species, Arctic char, sea raven and carp (Barton et al., 1995; Milligan, 1997; Vijayan et al., 1996; Liew et al., 2013b). This protein usage results in increased endogenous ammonia levels in the present study. Interestingly, carp that were fed a low ration retained more ammonia in plasma than the carp fed a high ration, even in carp that were not implanted with cortisol. Low plasma ammonia accumulation in high feeding fish indicated an enhanced detoxifying strategy by enhancing the excretion rate.

393 This is not only stimulated by cortisol but also by feeding itself (Liew et al., 2013b). In
394 addition, a transient plasma lactate elevation which returned to control levels afterwards was
395 seen in hypercortisol fish at 12h-PI. The lactate was likely being used for glycogenesis,
396 leading to a fast recovery of the plasma lactate level in cortisol implanted fish, and relatively
397 higher liver and muscle glycogen levels in cortisol implanted fish from 24h-PI onward.
398 Similar cortisol induced liver glycogenesis has been reported previously in Japanese eel
399 (Chan and Woo, 1978), rainbow trout (Hill and Fromm, 1968), killifish (Leach and Taylor,
400 1982) and common carp (Liew et al., 2013b).

401 Overall, plasma ions were maintained at a relatively stable level which was also
402 reflected in the stable plasma osmolality. Although plasma Na^+ showed a downwards trend in
403 cortisol treated fish, this Na^+ loss did not differ significantly. Feeding seems to contribute
404 substantially to a sufficient ion uptake, and therefore the loss of Na^+ caused by hypercortisol
405 was probably re-gained through Na^+ dietary intake (Na^+ content 5.88 mg/g pellet). Despite
406 these stable ion levels, gill NKA activity was greater in the high feeding fish than low feeding
407 fish. This increment in gill NKA activity had been reported previously and was shown to be
408 more associated with ammonia excretion during swimming rather than ion uptake per se
409 (Liew et al. 2013a) since feeding granted sufficient ion uptake via the gastrointestinal system
410 (Bucking and Wood, 2006; 2008; Bucking et al., 2010).

411 Cortisol stimulating gill NKA has been reported previously in cyprinids (Abo Hegab
412 and Hanke, 1984), cichlids (Dang et al., 2000), salmonids (Shrimpton and McCormick, 1999;
413 Wilson et al., 2002) and catfish (Babitha and Peter, 2010). Similarly, the presents study
414 confirmed that hypercortisol upregulated NKA activity in both gill and kidney in carp. In the
415 gill, a greater NKA activity was detected in carp fed a high ration. These fish also retained
416 less ammonia than the carp fed a low ration, while overall plasma ions and osmolality
417 remained stable. This probably reflected the additional role of gill NKA activity with

418 transporters involved in apical ammonia excretion such as the NH_4^+/Na^+ and/or Na^+/H^+
419 exchanger. Enhanced gill ammonia excretion, subsequently created a basal electro chemical
420 potential gradient to promote Na^+ influx by upregulating NKA activity and/or Na^+ channels
421 (Walsh, 1998; Salama et al., 1999; Wu et al., 2010; Zimmer et al., 2010; Liew et al., 2013a).
422 This link between Na^+ uptake and ammonia excretion, could also explain the increase of gill
423 H^+ ATPase activity which followed a similar trend as gill NKA activity with greater activity
424 observed in the carp fed a high ration.

425 High levels of cortisol did not only modulate gill ionoregulation, but also induced
426 increased kidney ionoregulation. An increase of kidney NKA activity was observed during
427 the first 24h-PI in low feeding fish, and at 24h-PI in high feeding fish. Overall, kidney NKA
428 activity detected in fish fed a low ration was comparable with the activity in fish fed a high
429 ration. In low feeding fish, a similar pattern as for NKA was observed for H^+ ATPase
430 activity, where hypercortisol increased kidney H^+ ATPase activity at the first 24h-PI.
431 However, no effect was observed for the H^+ ATPase activity in the fish fed a high ration and
432 the activities detected were much lower than in the low feeding fish. This again showed that
433 feeding provided sufficient ion intake in the high feeding fish, therefore the activity in the
434 kidney was maintained at a basal level. All ionoregulatory responses in the gill and the
435 immediate response to hypercortisol in the kidney explained why plasma ions remained
436 stable, with compromising strategies depending on food availability. Wood et al. (2009) and
437 Bucking et al. (2010) noted that even though the rate of kidney ionoregulatory activity was
438 lower than branchial activities, kidney ionoregulatory still played an important role in
439 maintaining basal ion levels. Nevertheless, a genomic response on the NKA mRNA
440 expression in the kidney of low feeding fish was only observed at later stage after cortisol
441 implantation.
442

443 *4.2 Gene expression*

444 The Rhesus glycoproteins examined in the present study showed no obvious upregulation
445 after a cortisol implant, and only Rhcg-1 was increased at 72h-PI in high feeding fish. The
446 Rhcg-1 upregulation in the gill likely helps to explain the low plasma ammonia level found in
447 high feeding fish by enhancing branchial ammonia excretion capacity when endogenous
448 ammonia levels were elevated. This is in parallel with previous studies reporting cortisol
449 regulates ionoregulatory and Rhesus glycoprotein gene expression in teleosts that may play a
450 key role in regulating expression of branchial ammonia excretion (Kiilerich et al., 2007;
451 Ivanis et al., 2008). This was clearly demonstrated by an *in vitro* gill culture of rainbow trout
452 challenged with cortisol, which activated the NH_4^+/Na^+ exchange and Rhesus glycoproteins
453 to increase ammonia transport in the gill epithelial cells (Tsui et al., 2009). Also, increased
454 ammonia excretion rates facilitated by Rhesus glycoproteins in parallel with increased plasma
455 cortisol levels were noticed in trout and cyprinid species when challenged with high
456 environmental ammonia (Sinha et al., 2013). This coincided with low plasma ammonia
457 accumulation; and stable ion levels and NKA and H^+ ATPase activities. The faster respons in
458 trasporter activities suggests that cortisol primarily stimulated non-genomic actions in carp to
459 cope with acute stress by activating the ionoregulatory metabolon in the gill. This confirms
460 our first and second hypothesizes that cortisol does trigger gill NKA and H^+ ATPase
461 activities, as well as Rhcg-1 expression in high feeding fish to facilitate ammonia excretion,
462 while stimulating kidney NKA and H^+ ATPase activities in low feeding fish for ion
463 reabsorption in common carp.

464 In the present study, the mRNA expression pattern of GR1, GR2 and MR in both gill
465 and kidney were differently expressed in response to hypercortisol. According to Teles et al.
466 (2013), gill is the primary action site of cortisol with an important role in osmoregulation.
467 They found that gill GR1, GR2 and MR levels were depressed in seabass 24h after being

implanted with cortisol, but the levels were upregulated thereafter at 240h-PI. In our study, the gill GRs and MR were not responding very sensitively even though plasma cortisol level was highly elevated during the first 12h-PI. In the low feeding fish, the only upregulation was detected for GR2 at 72h-PI. In the high feeding fish, GR2 and MR upregulation was observed at 72h-PI, followed by GR1 at 168h-PI. However, overall both feeding regimes exhibited an increasing trend of expression. Our finding is consistent with Yada et al., (2008) and Aruna et al., (2012a) who also found no significant difference in the GR1 and GR2 transcription at early stages, but later did find upregulation of GR2 and MR at 98h in trout and tilapia. They suggested the upregulation of GR2 and MR may play distinctive roles in hypo-and-hyper osmoregulatory mechanisms. The involvement of cortisol in ionoregulation via the GR1, GR2 and MR receptors were proven in gills of tilapia where co-localization of GR1, GR2 and MR transcripts with the mitochondria rich cells was observed during seawater acclimation (Aruna et al., 2012b).

Cortisol is produced and released from the head kidney to signal neuroendocrine stress responses in teleosts (Mommsen et al., 1999; Tort, 2011). Our results showed that kidney was the most sensitive organ that responded first to hypercortisol. GR2 downregulation was observed at 12h-PI in low feeding fish. This GR2 downregulation could reflect negative feedback due to the acute cortisol elevation: it would decrease tissue responsiveness to acute cortisol stimulation (Hontela et al., 1992; Takahashi et al., 2006) or a shock response of the pituitary-inter-renal-axis due to extreme hypercortisol level. However, the GR1, GR2 and MR started to be upregulated at 24h-PI to 72h-PI and returned to control at 168h-PI, while GR1 still remained upregulated at 168h-PI. In high feeding fish, upregulation of GR2 and MR responded at 24h-PI and remained upregulated up to 168h-PI. However, the levels of expression in low feeding fish were much higher at 24h-PI and lower at 168h-PI than in high feeding fish. Similarly, upregulation of GR1, GR2 and MR were also observed in seabass

493 after 24h-PI implanted with cortisol and GR1 continued to be upregulated untill 168h
494 (Vazzana et al., 2010). This probably indicates the relationship between corticoids and
495 increased NKA and H^+ ATPase activities for ionoregulation. Takahashi and Sakamoto (2013)
496 concluded that GRs may play a more important role in teleost osmoregulation than MR.
497 The liver is the tissue relevant for energetic and intermediary metabolism for all living
498 organisms. It is not surprising that extra energy expenditure was needed during stress to
499 compensate basal metabolic changes. Due to the cortisol elevation, our present results
500 indicate that hypercortisol induced liver protein mobilization for gluconeogenesis.
501 Additionally, this resulted in an increased plasma ammonia level. Extra energy mobilization
502 during stress is always associated with growth depression due to limited energy expenditure
503 for somatic growth. This phenomenon was referred to as 'high living cost' observed in
504 rainbow trout implanted with cortisol which not only depressed feeding performance and
505 growth rate, but also depressed aggressive behaviour (Gregory and Wood, 1998; 1999; De
506 Boeck et al., 2001).
507 Our results showed that the liver GR2 and MR were expressed in both feeding regimes
508 at 24h-PI and returned to control level afterwards and GR1 expression was only noticed in
509 high feeding fish. This expression pattern supports previous reports which noted that GR2 is
510 the most sensitive corticoid receptor in transmitting the stress signal, followed by the MR and
511 GR1 expression (Stolte et al., 2008a; 2008b). Liver GR2 and MR expression likely provided
512 energy mobilization for ionoregulation in gill and kidney. Cortisol promoted gluconeogenesis
513 and mobilized glucose to fuel gill metabolism, including activation of gill NKA activity for
514 hypo-osmoregulation, in tilapia acclimated to seawater as well (Vijayan et al., 1997;
515 Mommsen et al., 1999; McGuire et al., 2010). Also in our study increased plasma glucose
516 paralleled with high cortisol levels and higher NKA and H^+ ATPase activity and mRNA
517 expression in gill and kidney. Overall, the genomic expression of GR1, GR2 and MR mRNA

518 were upregulated after the hypercortisol peak in gill, kidney and liver which supported our
519 third hypothesis. However, in contrast to our earlier hypothesis, the receptors were not
520 upregulated to a larger extent in low feeding fish, except in kidney.
521
522 **5. Conclusion**
523 As a conclusion, we can summarize that cortisol plays an important role in regulating
524 immediate metabolic and ionoregulatory responses in carp during early stages of cortisol
525 elevation. A rapid upregulation of gill NKA and H^+ ATPase activities in carp fed a high
526 ration seem associated with ammonia excretion due to protein mobilization. In the kidney, the
527 upregulation of NKA and H^+ ATPase activities were more pronounced in fish fed a low
528 ration suggesting that enhanced ion reabsorption due to the limited dietary ion intake was
529 necessary. This enabled carp to maintain plasma osmolality and ions at a relative stable level.
530 Additionally, cortisol caused a repartitioning of energy for gluconeogenesis and glycogenesis.
531 Secondly, genomic responses to high cortisol happened in a later stage after the peak of
532 cortisol had passed, suggesting that these genomic responses require a more prolonged
533 cortisol elevation. In low feeding fish, kidney NKA mRNA expression was upregulated for
534 ionoregulation, while in high feeding fish gill Rhcg-1 was upregulated for ammonia
535 excretion. The GR1, GR2 and MR mRNA expression pattern showed tissue specific
536 responses with kidney as most sensitive organ, especially in low feeding fish. In combination
537 with the increased activity of ion transporters, this suggests a contribution of GR1, GR2 and
538 MR in renal ion reabsorption. Our results also revealed gill GRs and MR expression during
539 the plasma cortisol recovery period. This occurred simultaneously with the increase in
540 Rhesus glycoprotein mRNA expression but well after NKA and H^+ ATPase activities
541 increased. Further immunohistochemistry and *in situ* hybridization studies would be valuable

in order to address the relationship between corticoid receptors and ionoregulation in common carp.

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783 the interactive effects of feeding and high environmental ammonia on branchial
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List of tables

Table 1 Sequences of ionoregulatory primers used in the qPCR assay.

Genes	Primers sequence	Genbank Acc. No.
β-actin	FW: 5'-CGT-GAT-GGA-CTC-TGG-TGA-TG-3' RV: 5'-TCA-CGG-ACA-ATT-TCC-CTC-TC-3'	M24113
NKA	FW: 5'-AGG-TGG-ACA-ACT-CCT-CCC-TG-3' RV: 5'-ATA-CGA-CCC-ATG-ACA-GTA-CG-3'	JX570881
H^+ ATPase	FW: 5'-CTA-TGG-GGG-TCA-ACA-TGG-AG-3' RV: 5'-CCA-ACA-CGT-GCT-TCT-CAC-AC-3'	JX570880
Rhbg	FW: 5'- TCC-CAG-TTT-CCA-GGA-TGT-TC-3' RV: 5'-TGG-AAA-AAG-CCC-TGC-ATA-AG-3'	JX570877
Rhcg-1	FW: 5'-ATC-CTG-AAC-ATC-CTC-CAT-GC-3' RV: 5'-AAC-TTG-GCC-AGA-ACA-TCC-AC-3'	JX570878
Rhcg-2	FW: 5'-CAC-AAA-GCC-ACA-CAC-AGT-CC-3 RV: 5'-TCT-TTT-TCT-CGC-CGT-TCT-TG-3'	JX570879

791 **Table 2** Sequences of corticoid receptors primers used in the qPCR assay.

Genes	Primers sequence	Genbank Acc. No.
β-actin	FW: 5'-GCT-ATG-TGG-CTC-TTG-ACT-TCG-A-3' RV: 5'-CCG-TCA-GGC-AGC-TCA-TAG-CT-3'	M24113
GR1	FW: 5'-AGA-CTG-AGA-GGC-GGA-GCT-ACT-G-3' RV: 5'-GGC-GGT-GTT-GGC-TCC-AT-3'	AJ879149
GR2	FW: 5'-GGA-GAA-CAA-CGG-TGG-GAC-TAA-AT-3' RV: 5'-GGC-TGG-TCC-CGA-TTA-GGA-A-3'	AM183668
MR	FW: 5'-TTC-CCT-GCA-GAA-CTC-AAA-GGA-3' RV: 5'-ACG-GAC-GGT-GAC-AGA-AAC-G-3'	AJ783704

792

793

794 **Table 3** Liver protein, glycogen and lipid content (mg/g wet tissue) of common carp without

795 implants (control), and sham and cortisol implanted fish at different feeding regimes.

	Time	Low feeding			High feeding		
		Control	Sham	Cortisol	Control	Sham	Cortisol
Protein	12h	64.52±5.86 a	56.69±4.26 ab	42.71±4.51 b	79.32±4.78 a	65.15±5.74 ab	59.38±2.54 b
	24h	66.46±5.99 a	73.68±4.58 a	33.44±6.77 b	87.25±6.89 a	74.46±6.21 a	53.38±4.42 b +
	72h	57.33±9.42 ab	58.69±6.13 a	39.48±6.25 b	77.70±5.48	70.79±4.91	54.69±9.29
	168h	58.61±7.42	61.06±4.94	50.64±2.68	73.56±2.36 +	64.69±6.57	67.13±5.73 +
Glycogen	12h	53.89±3.81	61.14±2.97	64.38±5.74	85.06±4.44 +	79.02±4.35 +	98.78±8.28
	24h	53.67±2.64 a	62.51±3.63 ab	71.18±4.98 b	72.23±6.43 a +	83.91±4.22 ab +	103.52±7.35 b +
	72h	46.07±3.26 a	57.21±3.44 b	67.99±2.25 b	78.93±5.16 +	77.29±6.03 +	94.76±5.96 +
	168h	46.02±3.35 ab	41.19±3.11 a	54.02±3.57 b	74.44±5.81 +	63.63±2.31 +	76.40±6.88 +
Lipid	12h	23.58±2.71	21.51±1.98	25.31±3.51	29.07±2.58	27.62±1.78 +	31.68±1.79
	24h	24.14±2.41	26.74±1.68	21.96±2.31	33.69±2.83 +	31.36±3.07	28.52±2.77
	72h	22.49±2.89	18.79±1.67	22.51±3.04	31.96±4.07	24.29±2.45	28.66±2.41
	168h	24.72±2.71	21.59±1.98	18.73±1.71	27.67±2.42 ab	30.98±1.52 b +	22.61±2.78 a

A plus (+) indicates significant differences between feeding regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

796

797

798 **Table 4** Muscle protein, glycogen and lipid content (mg/g wet tissue) of common carp without

799 implants (control), and sham and cortisol implanted fish at different feeding regimes.

800

	Time	Low feeding			High feeding		
		Control	Sham	Cortisol	Control	Sham	Cortisol
Protein	12h	62.73±8.32	71.09±4.97	61.99±7.29	99.67±2.79 +	96.30±6.33 +	78.82±7.67
	24h	73.26±8.65	69.12±7.02	71.65±5.23	109.99±6.89 a +	97.57±6.39 a +	69.86±8.19 b
	72h	73.31±7.76	65.76±7.63	55.29±8.81	84.89±9.27	100.68±7.73 +	89.94±11.78 +
	168h	73.43±5.54	75.55±9.31	67.24±12.37	91.51±7.86	104.86±3.63	100.77±5.11
Glycogen	12h	70.68±5.84	57.97±4.03	65.43±4.82 AB	70.79±4.71	79.91±7.92 +	92.49±7.06 AB +
	24h	61.38±6.37	66.67±5.13	74.91±7.35 A	72.04±5.07	78.62±9.55	103.69±7.45 A +
	72h	60.27±5.71	56.87±4.15	67.23±5.72 AB	73.11±3.82 a	89.82±5.57 ab +	104.49±5.71 b A +
	168h	54.65±2.99	57.63±3.96	53.82±4.42 B	62.78±5.58	67.17±5.81	74.99±6.91 B +
Lipid	12h	18.34±2.05	16.48±1.24	16.09±1.24	21.66±2.90 +	22.88±1.72 +	18.67±1.24
	24h	17.98±1.50	16.16±0.93	15.49±0.88	20.46±2.54	20.06±1.72	18.11±1.19
	72h	16.01±1.19	14.25±1.09	14.46±0.79	19.83±2.43	22.47±2.71 +	20.21±1.61 +
	168h	15.95±1.34	13.64±0.54	14.46±1.08	22.42±1.69	20.04±2.65 +	18.40±1.55

A plus (+) indicates significant differences between feeding regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

801

802

803 **List of figure captions**

804

805 **Fig. 1** Plasma cortisol levels of control, sham implanted and cortisol implanted common carp
806 fed 0.5% BW (A) and 3.0% BW (B) daily throughout 168h-PI. Small letters indicate
807 significant differences between cortisol treatments. Capital letters indicate significant
808 differences between time intervals.

809

810 **Fig. 2** Plasma glucose (A and C) and lactate (B and D) levels of control, sham implanted and
811 cortisol implanted common carp fed 0.5% BW and 3.0% BW daily throughout 168h-PI.
812 Small letters indicate significant differences between cortisol treatments. Capital letters
813 indicate significant difference between time intervals. A plus (+) indicates significant
814 differences between feeding regimes.

815

816 **Fig. 3** Plasma ammonia (A and C) and urea (B and D) levels of control, sham implanted and
817 cortisol implanted common carp fed 0.5% BW and 3.0% BW daily throughout 168h-PI.
818 Small letters indicate significant differences between cortisol treatments. Capital letters
819 indicate significant differences between time intervals. A plus (+) indicates significant
820 differences between feeding regimes.

821

822 **Fig. 4** Plasma osmolality (A and E), sodium, Na^+ (B and F), chloride, Cl^- (C and G) and
823 potassium, K^+ (D and H) levels of control, sham implanted and cortisol implanted common
824 carp fed 0.5% BW and 3.0% BW daily throughout 168h-PI. Small letters indicate significant
825 differences between cortisol treatments. Capital letters indicate significant differences
826 between time intervals. A plus (+) indicates significant differences between feeding regimes.

827

828 **Fig. 5** Gill (A and C) and kidney (B and D) Na^+/K^+ ATPase activity levels of control, sham
829 implanted and cortisol implanted common carp fed 0.5% BW and 3.0% BW daily throughout
830 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital
831 letters indicate significant differences between time intervals. A plus (+) indicates significant
832 differences between feeding regimes.
833
834 **Fig. 6** Gill (A and C) and kidney (B and D) H^+ ATPase activity levels of control, sham
835 implanted and cortisol implanted common carp fed 0.5% BW and 3.0% BW daily throughout
836 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital
837 letters indicate significant differences between time intervals. A plus (+) indicates significant
838 differences between feeding regimes.
839
840 **Fig. 7** mRNA Na^+/K^+ ATPase and H^+ ATPase expression levels in the gill (A and C) and
841 kidney (B and D) of common carp fed 0.5% BW and 3.0% BW daily throughout 168h-PI
842 with cortisol implants. Samples from the control and sham implanted groups were randomly
843 selected (n=8) and served as control for the cortisol implanted fish (n=4-5). An asterisks (*)
844 indicates significant differences compared to the control group. A plus (+) indicates
845 significant differences between feeding regimes.
846
847 **Fig. 8** mRNA Rhesus glycoprotein (Rhbg, Rhcg-1 and Rhcg-2) expression levels in the gill
848 of common carp fed 0.5% BW and 3.0% BW daily throughout 168h-PI with cortisol
849 implants. Samples from the control and sham implanted groups were randomly selected (n=8)
850 and served as control for the cortisol implanted fish (n=4-5). An asterisks (*) indicates
851 significant differences compared to the control group.
852

Fig. 9 mRNA glucocorticoid receptor-1 (GR1), glucocorticoid receptor-2 (GR2) and minerolocorticoid (MR) expression levels in the gill (A and D), kidney (B and E) and liver (C and F) of common carp fed 0.5% BW and 3.0% BW daily throughout 168h-PI with cortisol implants. Samples from the control and sham implanted groups were randomly selected (n=8) and served as control for the cortisol implanted fish (n=4-5). An asterisks (*) indicates significant differences compared to the control group. A plus (+) indicates significant differences between feeding regimes.

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Figure 1: Plasma cortisol

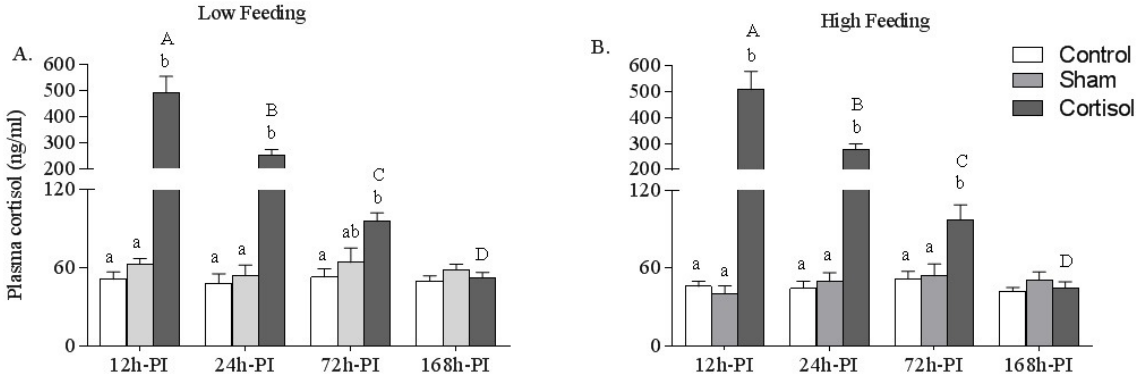

Figure 2: Plasma glucose and lactate

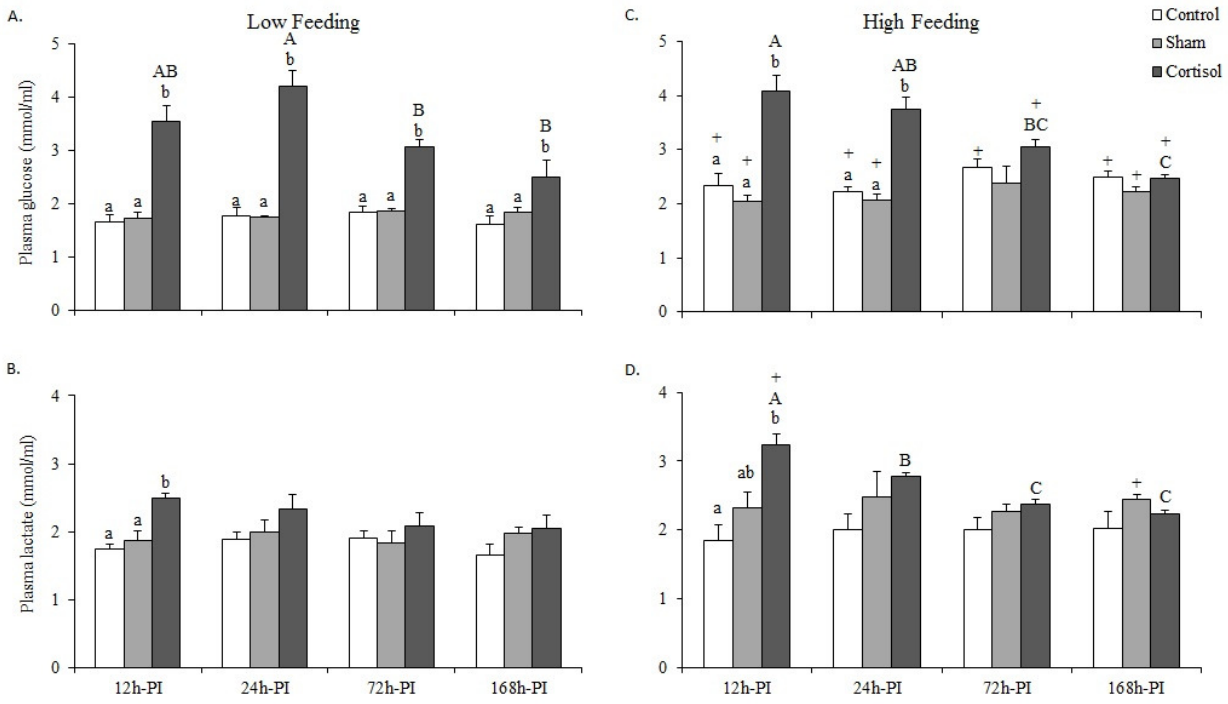

Figure 3: Plasma ammonia and urea

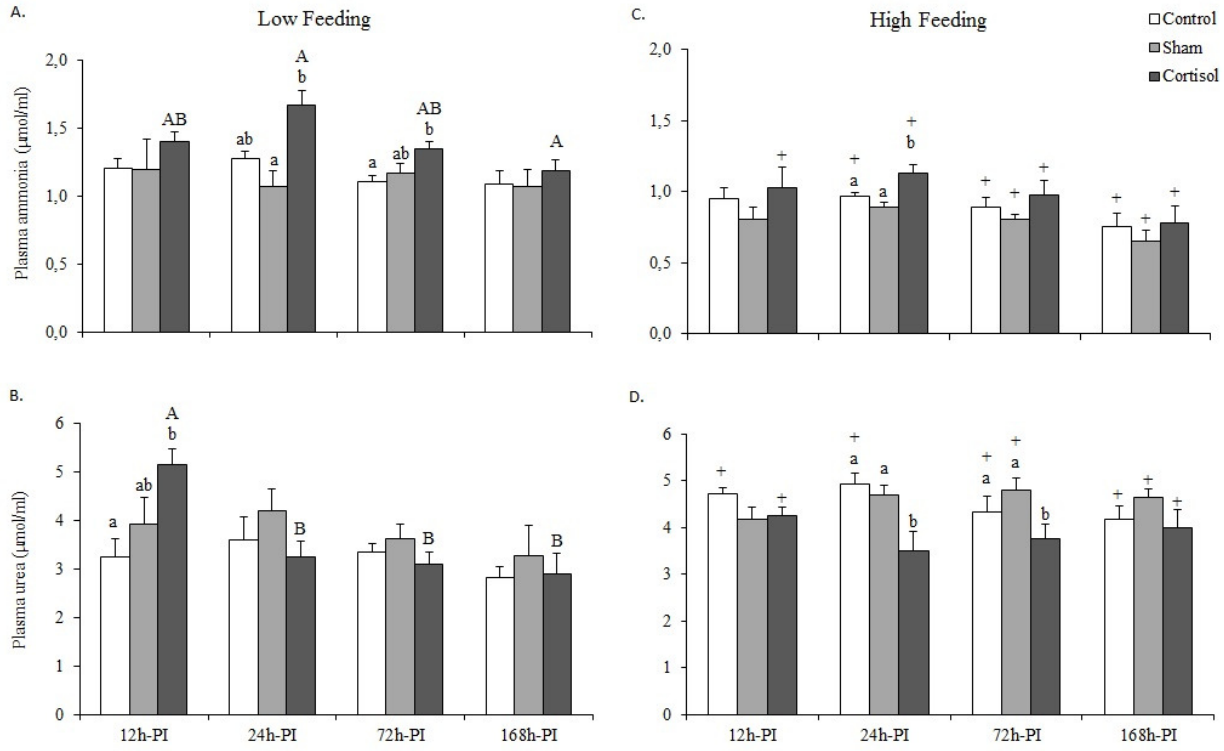

Figure 4: Plasma osmolality and ions

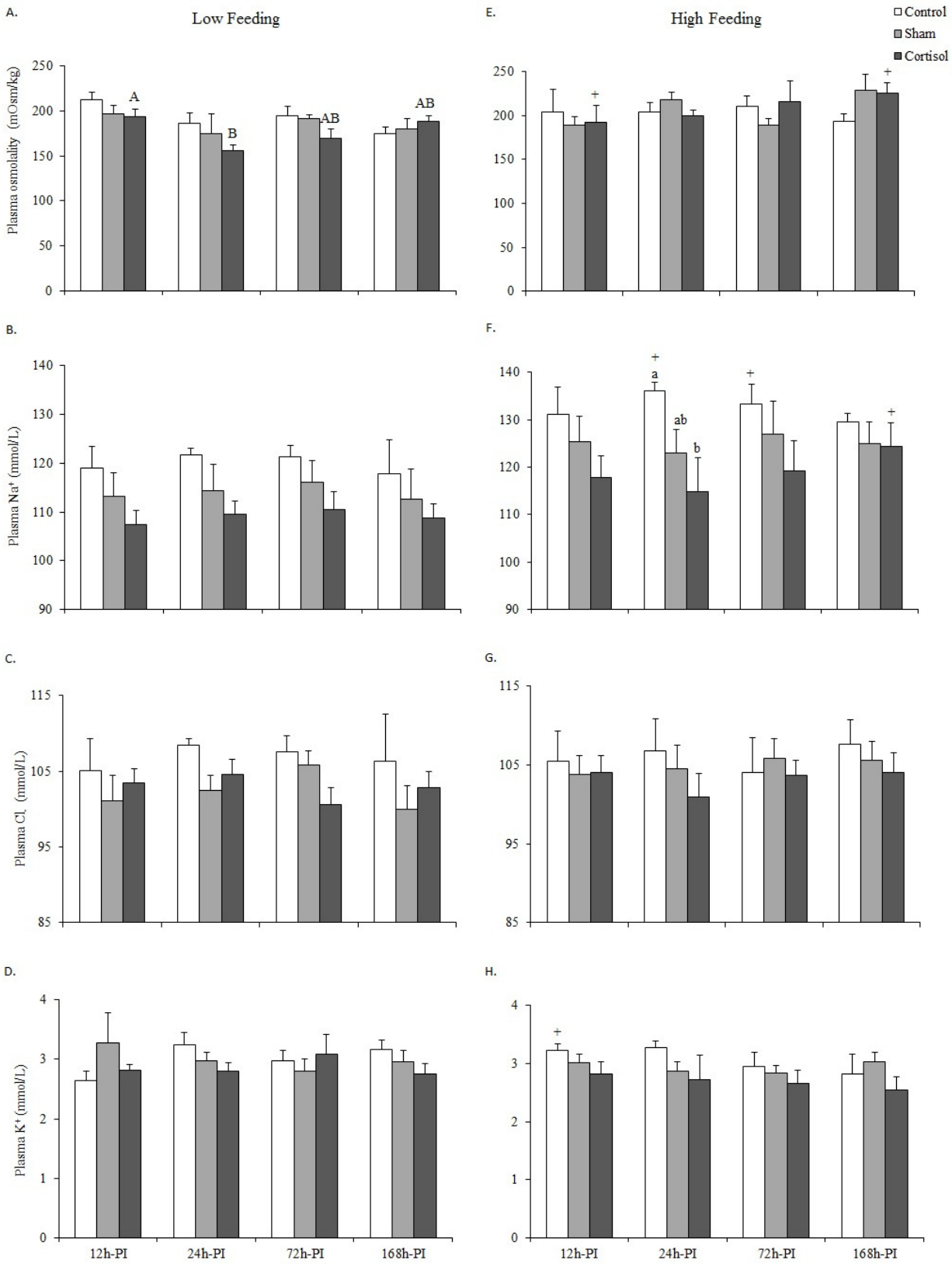

Figure 5: Gill and kidney NKA

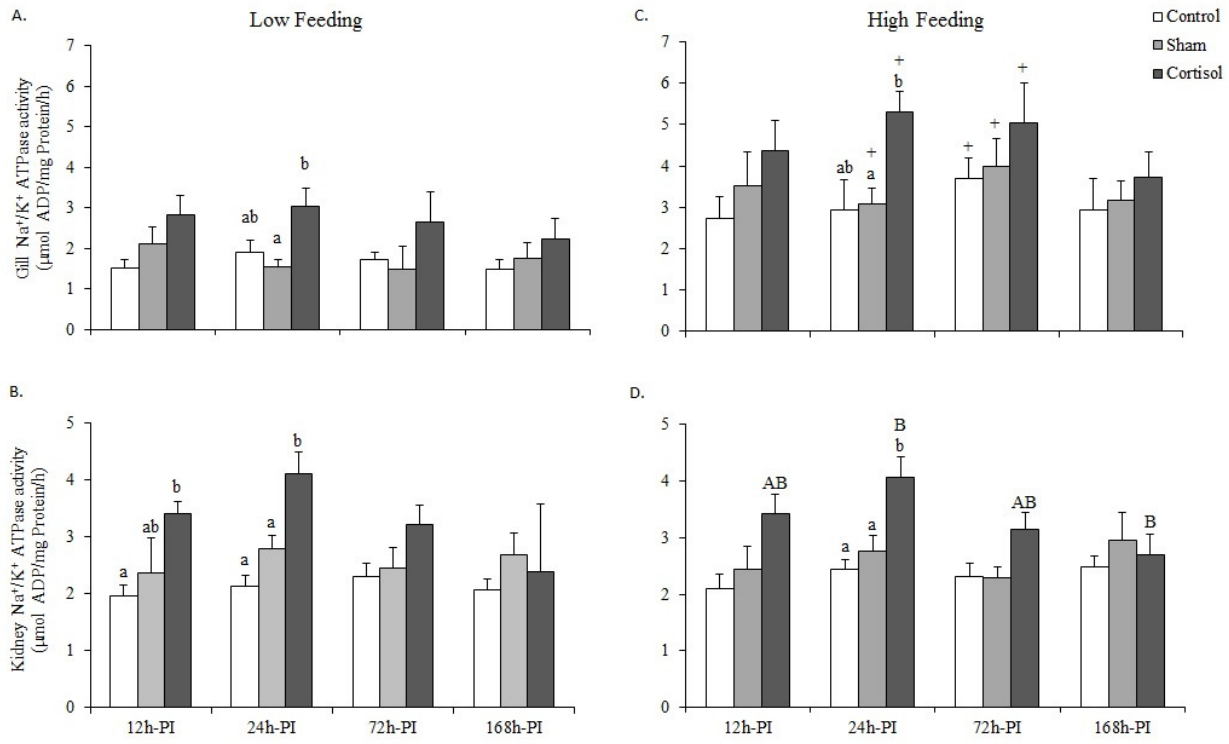

Figure 6: Gill and kidney H^+ ATPase

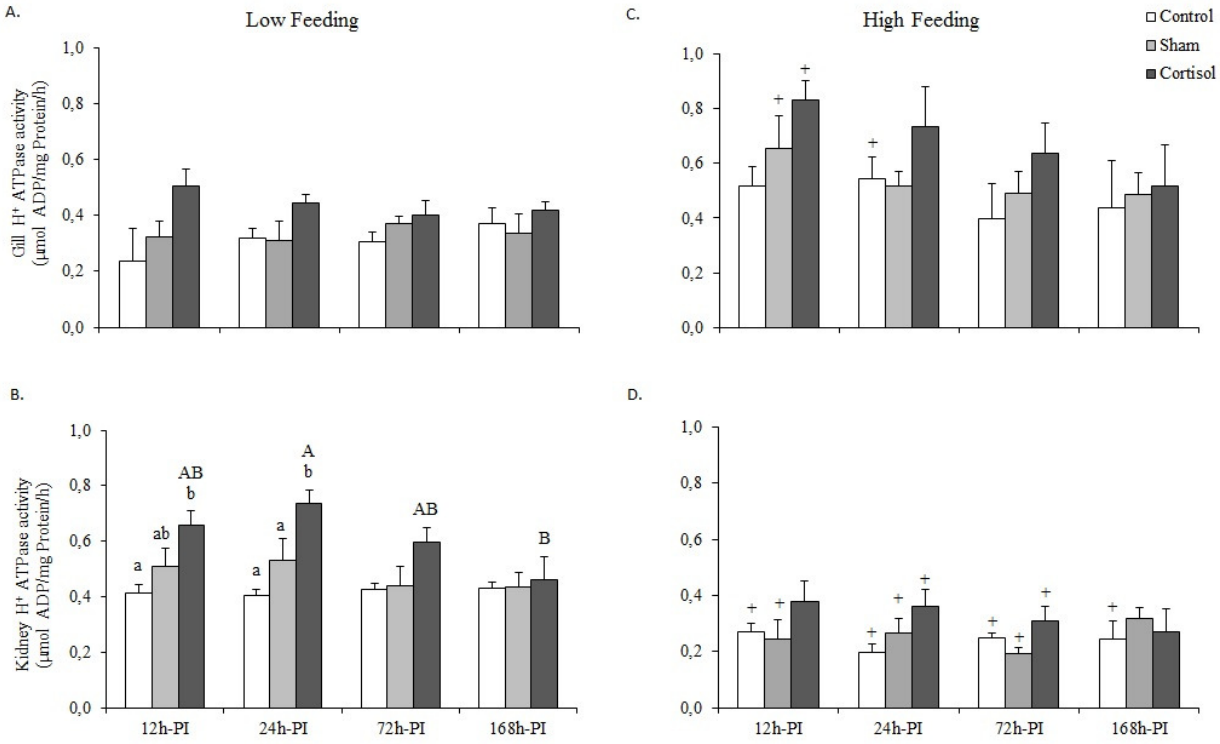

Figure 7: Gill and Kidney NKA and H^+ ATPase expression

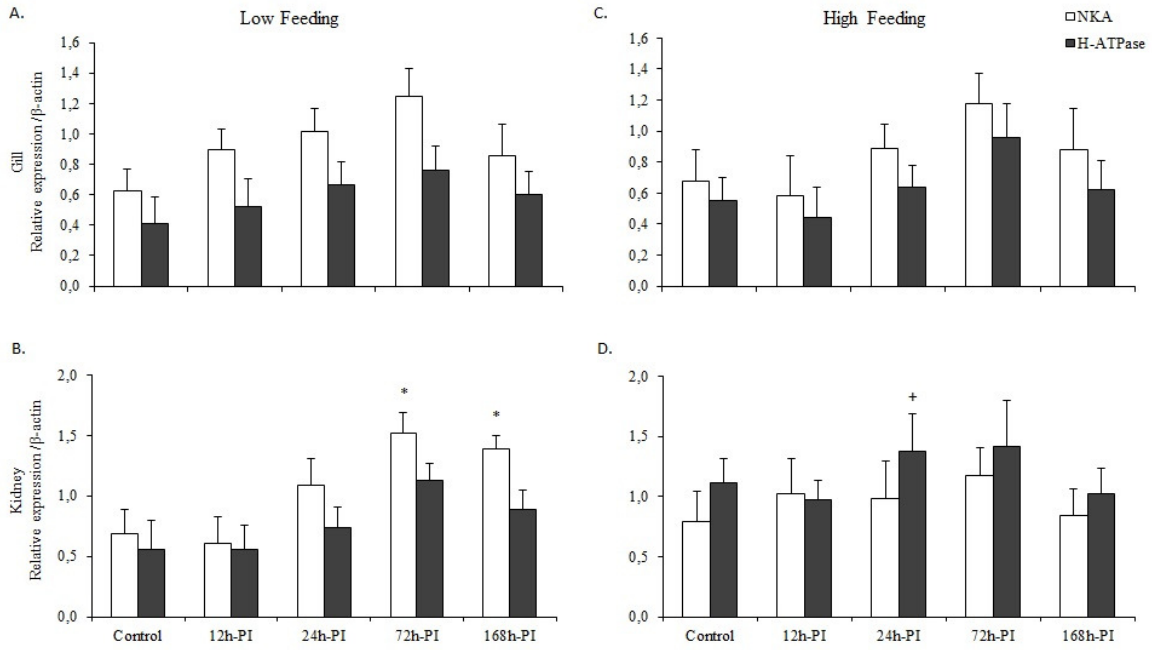

Figure 8: Gill Rhesus glycoprotein expression

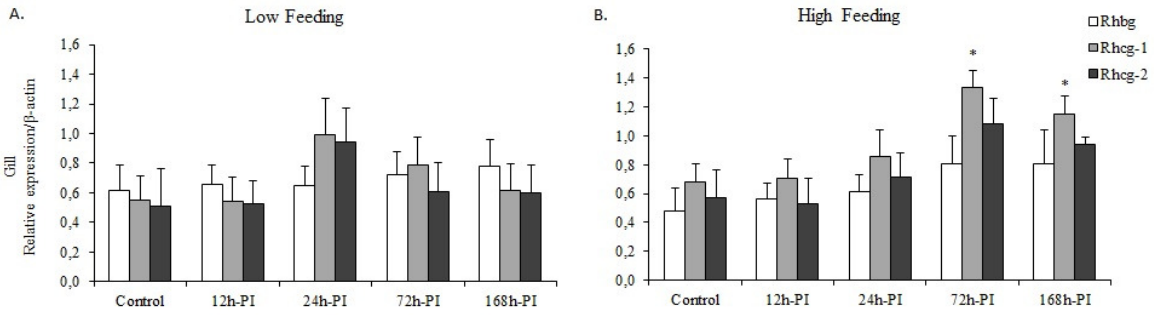

Figure 9: Gill, kidney and liver corticoid receptors expression (GR1, GR2, MR)