Effect of Injection of Lipopolysaccharides from *Aeromonas salmonicida* on some Aspects of Cod (*Gadus morhua*) Immunity and Appetite Hormones

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EFFECT OF INJECTION OF LIPOPOLYSACCHARIDES FROM *AEROMONAS SALMONICIDA* ON SOME ASPECTS OF COD (*GADUS MORHUA*) IMMUNITY AND APPETITE HORMONES

by

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ABSTRACT

Pérez-Casanova, J.C., Hamoutene, D., Volkoff, H., Mabrouk, G., Samuelson, S., and Burt, K. 2010. Effect of injection of lipopolysaccharides from *Aeromonas salmonicida* on some aspects of cod (*Gadus morhua*) immunity and appetite hormones. Can. Tech. Rep. Fish. Aquat. Sci. 2878: v + 11 p.

In vertebrates, including fish, strong interactions exist between immune and neuroendocrine systems. The effects of disease on the feeding of fish have only began to be explored and studies on the role that appetite-regulating hormones play in fish feeding during an infection are scarce. Atlantic cod is a species of considerable economic importance in the North Atlantic and in recent years experimental farming has been successfully carried out in Canada. In this context, studies on the interaction of feeding and the immune system of Atlantic cod are highly relevant. Thus, we investigated the effects of lipopolysaccharide (LPS) from *Aeromonas salmonicida* on the mRNA expression of the appetite regulating peptides ghrelin, neuropeptide Y (NPY) and cocaine and amphetamine-regulated transcript (CART) of Atlantic cod juveniles. In addition, we measured the effects of LPS on the fish cellular immune responses (respiratory burst, RB) of head kidney and whole blood leukocytes and stress responses in juvenile Atlantic cod (*Gadus morhua* L.). Juvenile fish were injected intraperitoneally with either saline or saline containing LPS and sampled at 24h and 48 h post exposure. Results indicate that intraperitoneal injections of LPS (5 ng g^{-1}) may have a negative effect on juvenile Atlantic cod innate immune response as indicated by the decrease in RB response of whole blood and head kidney leukocytes. At doses of 5 ng g^{-1}, LPS did not affect the expression of appetite regulating factors or the physiological stress response of Atlantic cod. These results provide researchers with information on potential reactions of Atlantic cod to LPS and establish the basis for future research on this subject.

RÉSUMÉ

Pérez-Casanova, J.C., Hamoutene, D., Volkoff, H., Mabrouk, G., Samuelson, S., and Burt, K. 2010. Effect of injection of lipopolysaccharides from *Aeromonas salmonicida* on some aspects of cod (*Gadus morhua*) immunity and appetite hormones. Can. Tech. Rep. Fish. Aquat. Sci. 2878: v + 11 p.

D'importantes interactions existent entre le système immunitaire et le système endocrinien des Vertébrés y compris des poissons. Les effets d'une pathologie sur l'appétit et la nutrition commencent à peine à être étudiés mais peu d'études ont été complétées chez le poisson. La morue d'Atlantique est une espèce d'importance considérable dans l'Atlantique du Nord et dont l'aquaculture se développe rapidement. Pour toutes ces raisons, nous avons étudié le lien entre certaines réponses immunitaires et l'appétit des morues. Nous avons injecté des morues avec des Lipopolysaccharides (LPS) d'Aeromonas salmonicida et étudié l'effet de cette injection sur l'expression génétique de peptides régulant l'appétit (Ghrelin, NPY et CART) ainsi que sur les réponses des cellules du système immunitaire inné et des niveaux de stress. Nous avons injecté des morues juvéniles avec des solutions salines et de LPS et procéder a des prélèvements 24 heures et 48 heures après injection. Nos résultats indiquent que les injections de LPS ont un effet négatif sur les réponses des cellules immunitaires. Aux doses de 5 ng g-1, les injections de LPS n'ont pas d'effet sur l'appétit et le stress des poissons. Nos résultats apportent de l'information sur les réactions de la morue aux injections de LPS pouvant ainsi établir une base pour de futures recherches dans le domaine.

INTRODUCTION

Bacterial lipopolysaccharides (LPS) are structural components of the external layer of the outer membrane of Gram-negative bacteria and are considered to be the cause for their ability to induce disease (Iliev et al. 2005; Swain et al. 2008). LPS treatment in mammals has been shown to induce a number of biological effects including shock, neutropenia, intravascular coagulation, leucocytosis, leucopemia and others, although these responses vary with species and with the dose of LPS (Swain et al. 2008). In fish and other aquatic vertebrates, the effects of LPS include pathological, physiological, immunological, immuno-endocrinological and neuro-immunological responses (Balm et al. 1993; Balm et al. 1995; Pepels et al. 2004; Swain et al. 2008). For example, LPS is a potent stimulator of the cellular immunity in fish and in vivo and/or in vitro exposure to LPS results in stimulation of the respiratory burst response (RB; the production of reactive oxygen species by the cell upon stimulation) of head kidney and circulating leukocytes (Swain et al. 2008). Injecting fish with LPS from a fish -specific pathogen (e.g., *Aeromonas salmonicida*) is a commonly non-lethal procedure used to induce an immune reaction against bacterial surface molecules (Hoeger et al. 2004), thus allowing investigators to study the in vivo effects of a toxicant/stress on reactivity against a threatening disease while avoiding animal death.

Strong interactions exist between immune and neuroendocrine systems of fish and other vertebrates. Following an immune challenge such as a bacterial infection or inflammation, sick animals display a set of non specific symptoms collectively called the acute phase response. These include reduced locomotion, lethargy and reductions in food intake. Acute phase responses have been observed following LPS treatment. LPS most probably exerts its actions through effects on the central nervous system (CNS); however, the mechanisms involved in these actions are not completely understood (Berczi 1998).

Feeding and energy homeostasis in vertebrates appears to be regulated by specific regions of the brain, namely the hypothalamus and the ventral telencephalon in particular (Peter 1979; Valassi et al. 2008); these regions produce peptides that either increase (orexigenic factors) or decrease (anorexigenic factors) feeding. In addition to the CNS, the control of food intake also involves factors produced by peripheral organs such as the gastrointestinal tract (GIT) and adipose tissue. Orexigenic factors include neuropeptide Y (NPY), orexin and ghrelin, whereas cocaine and amphetamine-regulated factor (CART) is an example of anorexigenic factor. Several of these appetite-regulating factors have been cloned and characterized in fish, suggesting that the control of food intake is conserved among vertebrates (Valassi et al. 2008; Volkoff et al. 2009). As feeding is under the control of the CNS, the reduction in appetite during an infection may be the result of the interactions between LPS and brain regions that regulate appetite.

The effects of disease and bacterial infection on the feeding of fish have only begun to be explored and studies on the role that appetite-regulating hormones play in fish feeding during an infection are scarce. In goldfish (*Carassius auratus* L.), IP injections

of LPS induce a dose dependent decrease in food consumption (Volkoff and Peter 2004) and high doses of LPS induce a decrease in the brain mRNA expression of NPY and an increase of CART, cholecystokinin and corticotrophin-releasing factor. In tilapia, LPS treatment induces changes in brain corticotropin-releasing factor immunoreactivity (Pepels et al. 2004).

Atlantic cod is a species of considerable economical importance in the North Atlantic. In recent years experimental Atlantic cod farming has been successfully carried out in Canada, Iceland, Norway and Scotland (Chambers and Howell 2006) and may become an economically viable industry in the foreseeable future. In this context, studies on the regulation of feeding and the immune system of Atlantic cod are highly relevant (Magnadottir et al. 2001). Previous studies on the immune system of Atlantic cod have highlighted some unusual features compared to other teleost species, in particular the apparent inability to produce a specific antibody response (Magnadottir et al. 2001), suggesting that innate immunity is of most importance for this species.

In this study, we investigated the effects of intraperitoneal (IP) LPS injections on the mRNA expression of the appetite regulating peptides ghrelin, NPY and CART as well as on the innate immune and stress responses in juvenile Atlantic cod (*Gadus morhua* L.). Results are discussed with respect to developing a better understanding of immune cell reactions and the interactions between immune reactivity and appetite regulation in Atlantic cod.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Juvenile Atlantic cod were obtained from the Joe Brown Aquaculture Research Building (JBARB) of the Ocean Sciences Centre, Memorial University of Newfoundland and were the progeny of cultured broodstock. Prior to the experiment, fish were allowed to recover from netting, transport and handling stress for a period of 2-3 months. After this acclimation period, fish (53.40 ± 15.97 g mean wet mass) were randomly assigned to each one of 4 different delimited areas (12 fish per quadrant) of a single 750 L square tank as follows: quadrant 1: saline injected fish, quadrant 2: LPS injected fish, quadrant 3: extra fish, and quadrant 4: control non-injected fish. During the acclimation period and during the experiment (lasting 3 days), all fish were manually fed sinking 3 mm marine pellets (Skretting, NB, Canada) until apparent satiation was reached.

FISH EXPOSURE AND SAMPLING

On day 1 of the experiment, individual fish were carefully netted out of the tank and quickly IP-injected, according to Seternes et al. (2001), using 1 cc U-100 syringes (Becton Dickinson and Company, Franklin Lakes, NJ, U.S.A.) with 200 µl of either fish saline or saline containing 0.5 ng g^{-1} of LPS from *Aeromonas salmonicida*. LPS was

prepared as described in Banoub et al. (2004). All fish from each group were sampled in 2 consecutive days starting 24 h after injections. During sampling, fish were carefully netted out of the tank and blood samples were taken from the caudal vein using previously heparinized (100 U ml^{-1} heparin; Sigma Aldrich, St Louis, MO, U.S.A.) 1 cc U-100 syringes. Fish were then killed by head severance and the brain removed. The hypothalamus and telencephalon areas of the brain were then excised and individually placed in 1.5 ml microcentrifuge tubes containing 4 volumes of RNAlater and stored at -20 °C until further processing. Gut samples were also taken and stored in RNAlater.

RESPIRATORY BURST STUDIES

Blood samples were collected, diluted in isolation buffer (MCHBSS + Alsever's buffer) (Crippen et al. 2001), and kept on ice for respiratory burst (RB) response evaluation. Dissociated head-kidney leucocytes were obtained by pressing through a nylon screen (50-60 µm) in the presence of isolation buffer. The cell suspension was layered on a Percoll gradient 33/51% in phosphate buffered saline and NaCl 1.8% centrifuged at 400xg for 30 minutes according to Stenvik et al. (2004). The cells at the interface were recovered and kept on ice until used for RB measurements. Respiratory burst was quantified in 4 samples per experimental group per day using flow cytometry to measure the intracellular hydrogen peroxide production following activation with 1 µg/mL of phorbol myristate acetate (PMA) based on the procedure previously described by Bass et al. (1983). The assay depends upon the cell incorporating 2'–7' dichlorofluorescin diacetate (DCFH-DA), a nonfluorescent molecule which is oxidized to fluorescent DCF. Briefly, isolated circulating leukocytes (or whole blood) were incubated with DCFH-DA (5 µM) at room temperature and baseline fluorescence levels were measured. PMA was added and fluorescence was measured immediately and 10 min after cell stimulation. Between 10 000 and 30 000 events were included in the analysis of every blood sample. For each sample, a stimulation index was determined as the ratio of fluorescence of PMA stimulated cells (10 min) to that of cells at ''time 0''. Cellular debris was excluded from the analysis by raising the forward-scatter threshold only minimally.

STRESS STUDIES

Total plasma cortisol and lactic acid were measured on samples of control fish and fish IP-injected with saline or LPS, 48 h post injection only. Lactic acid analyses were carried out on a Beckman LX automated analyzer at the hematology/biochemistry laboratory of the General Hospital, St John's, Newfoundland. Total plasma cortisol was measured using a Unicel Dxl800 Access® immunoassay analyzer with a Cortisol immunoassay kit (Beckman).

mRNA EXPRESSION STUDIES

Hypothalamus, telencephalon and gut samples were thawed on ice and the RNAlater removed using a micro-pipetor. Total RNA was then extracted using the TRIzol Reagent (Invitrogen Canada Inc., Burlington, ON) method following the manufacturer's instructions. One µl of the final total RNA solution was diluted 70-fold in RNase free water and the RNA concentration was measured spectrophotometrically using a GeneQuant Pro III RNA/DNA calculator (Biochrom Ltd., Cambridge, UK).

Transcript (mRNA) expression levels of CART, NPY, ghrelin and elongation factor 1α (EF-1α, used as a normalizer gene) in the hypothalamus, telencephalon and gut samples, respectively, were quantified by quantitative RT-PCR using SYBR Green I dye chemistry and the 7500 Real Time PCR system (Applied Biosystems, Foster City, CA). EF-1 was tested and found to be stably transcribed in all experimental groups.

The sequences of the primers used in gene expression analysis were based upon Atlantic cod sequences for CART, NPY, ghrelin and EF-1α that were obtained from GenBank (Benson et al. 2008; Table 1). Prior to use, multiple primer pairs for each gene of interest and the normalizing gene were tested. Dissociation curves showed a single peak and no primer dimer products were present in the no-template controls. Amplification efficiency was also calculated for two random samples from the experimental groups using a 5 point 1:4 dilution series starting with 10 ng of cDNA, with the reported value (Table 1) being the average of the three groups (control + saline + LPS groups). Multiple candidate qRT-PCR primer pairs were tested for genes of interest, and primer pairs with the best performance (e.g., single PCR product, no primer dimer, high amplification efficiency) were selected for use. Amplicon sizes are approximately 100bp for all primer pairs (Table 1).

First-strand cDNA was synthesized for 4 samples per experimental group per sampling day from 1 µg of total RNA using the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. This kit includes removal of genomic DNA prior to cDNA synthesis.

PCR amplification was performed with the 7500 PCR Detection System (Applied Biosystems, Foster City, CA) in a 25 µl reaction using 1 µl (10 ng) of cDNA, 50 nM each of forward and reverse primer and 1× Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The real-time analysis program consisted of 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The expression levels of the genes of interest were normalized to EF-1α. The genes of interest and the normalizer gene were tested in duplicate and the fluorescence threshold cycle (CT) determined using the 7500 PCR Detection System SDS Software Relative Quantification Study Application (Version 1.2.3). The relative starting quantity (RQ) of each transcript was determined using the $2^{-\Delta\Delta C_T}$ method, using the individual with the lowest gene of interest expression (highest CT value) as calibrator and assuming 100% efficiencies.

Table 1. Primers used in quantitative reverse transcription-polymerase chain reaction.

Gene of Interest	Primers	Nucleotide Sequence (5' → 3')	GenBank Acc. No.	Efficiency (%)	Amplicon Size (bp)
Elongation Factor 1 alpha	EF1α Forward	TGAAGTCCGTTGAGATGCAC	DQ402371	96.45	87
	EF1α Reverse	TGATGGAGACGTTCTTGACG			
Cocaine and Amphetamine Regulated Transcript	CART Forward	AGGTCCAAACTTGACCAACG	DQ167209	100.84	134
	CART Reverse	ACTGCTCTCCGATATCACACG			
Neuropeptide Y	NPY Forward	ACTCCGCATTGAGGCACTAT	AY822596	96.96	108
	NPY Reverse	TTTCCTTCAGCACCAGCTCT			
Ghrelin	Ghrelin Forward	ATCCTCCTCCTTTGCTCTCTG	EU128174	99.40	90
	Ghrelin Reverse	TTTGTCCAGGGGTTTGTGAG			

STATISTICAL ANALYSIS

Two-way ANOVAs were used to analyze the effects of treatment and sampling day on the parameters of interest. Before parametric test were performed, all data were analyzed for homogeneity of variances and the normality of the residuals. When parametric tests were not appropriate, Kruskal-Wallis ANOVAs were used to analyze the data. One way ANOVAs were used to compare total plasma cortisol and lactate levels between groups. For all analyses, P values < 0.05 were considered to be significant. When differences between groups were significant,Tukey's multiple comparison tests were used.

RESULTS

IP injections of *A. salmonicida* LPS at a dose of 0.5 ng g^{-1} had no effect ($P > 0.05$) on the RB of whole blood 24 h after the injection; however, a significant ($P = 0.032$) decrease in RB of whole blood in fish from the LPS group was observed 48 h after injection (Table 2). With regards to the RB of HK leukocytes, a significant ($P = 0.036$) difference between the LPS group and the saline group was detected 24 h post injection, although no difference` between the control group and either the saline or LPS groups was evident. When analyzed 48 h post injection, there was no ($P > 0.05$) difference in the RB of HK leukocytes between any of the experimental groups (Table 2).

Table 2. The respiratory burst response of whole blood and head kidney leukocytes 24 h and 48 h post intraperitoneal injection with saline or 0.5 ng g^{-1} of LPS from *Aeromonas salmonicida*. Different letters denote statistical significance ($P < 0.05$; $n = 4$).

	Whole Blood		Head Kidney	
	24 h	48 h	24 h	48 h
Control	4.76 ± 1.88^{a}	6.67 ± 1.37^{a}	3.97 ± 1.43^{ac}	3.47 ± 0.37^{a}
Saline	4.94 ± 0.84^{a}	5.38 ± 0.88^{a}	5.63 ± 4.62^{ab}	4.23 ± 0.83^{a}
LPS	5.10 ± 1.67^{a}	4.91 ± 0.61^{b}	2.70 ± 0.61^{c}	3.28 ± 0.89^{a}

LPS injections induced a significant ($P < 0.05$) decrease in lactate. An increasing trend was seen for total serum cortisol concentrations 48 h post injection, but this increase was not statistically significant ($P > 0.05$; Fig. 1).

LPS injections had no significant ($P > 0.05$) effect on the expression of CART, NPY or ghrelin either 24 or 48 h post injection (Fig. 2).

DISCUSSION

This lack of immunological memory suggests that the innate immune factors are very important in securing immune defense. Furthermore, innate immune parameters such as the RB of leukocytes are the first line of defense against pathogens and are known to respond faster to antigens than specific immune parameters (Hoeger et al. 2004). Our results show that the RB of whole blood significantly ($P < 0.05$) decreases in the LPS injected group at 48 h post-injection, which suggests that LPS has a significant impact on immune cell reactivity. This is in contrast with results of previous studies performed in this species; for example, Sørensen et al. (1997) demonstrated that the in vitro production of reactive oxygen species was significantly increased by the stimulation of the cells with 10 µg ml^{-1} LPS from *Vibrio anguillarum* (Sørensen et al. 1997). The difference between our results and those reported by Sørensen et al. (1997) may be due to the differences in the size of the fish used in the experiments (600 - 1000 g in the Sørensen study vs. ~ 50 g in our study). This hypothesis is likely in view of studies showing that in Atlantic cod, innate immune parameters are clearly influenced by the size of the fish (Magnadottir et al. 1999). Head kidney RB was affected by the injection itself as indicated by the change due to saline injection. When compared to saline injected fish, HK RB of LPS injected fish is lower only at 24 h. Sarmento et al. (2004) found that in sea bass (*Dicentrarchus labrax*), the RB of head kidney leukocytes, although initially increased, was decreased after 24 h of incubation with LPS. Time might be a factor in these response patterns, our results suggest a delayed reaction of blood leucocytes compared to HK with RB returning to normal in HK after 48 hours.

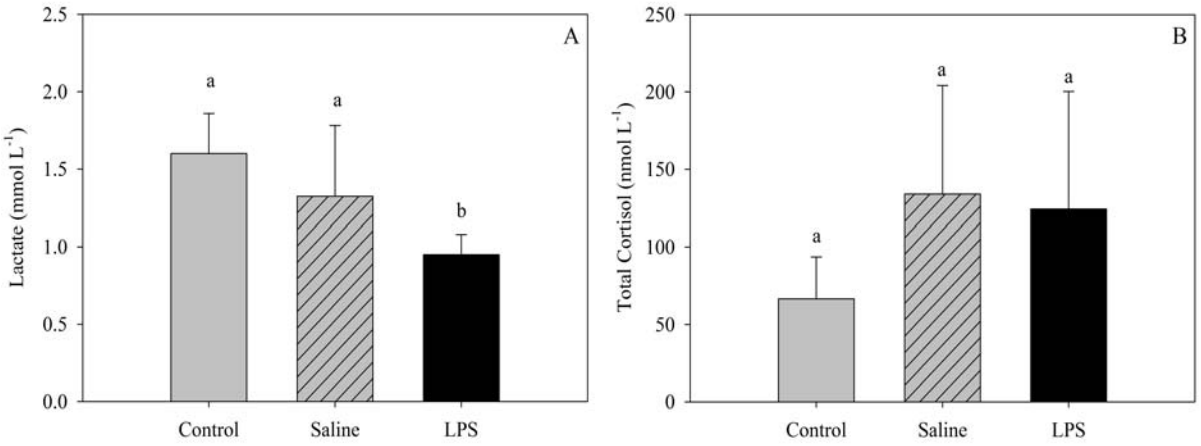

Figure 1. Stress response of juvenile Atlantic cod I48 h after injection with 0.5 ng g^{-1} of LPS from *Aeromonas salmonicida*. Levels of serum lactate (A) and total serum cortisol (B) were analyzed by a one way ANOVA. Dissimilar letters denote statistical difference ($P > 0.05$; $n = 6$) within each parameter. Values are means ± st. dev.

With regards to the stress response, we measured the levels of serum lactate and total cortisol 48 h after injection. Cortisol is considered a reliable indicator of the stress response in fish (Barton 2002), particularly in Atlantic cod and other gadoids (Hosoya et al. 2007; Pérez-Casanova et al. 2008). An increase in lactate level has been suggested to be part (in terms of carbohydrate metabolism) of a common pattern of stress response in fish (Pacheco and Santos 2001; Simontacchi et al. 2008). Our results show that serum lactate significantly ($P < 0.05$) decreases in the LPS-injected group 48 h post-injection. Although levels of lactate are commonly increased shortly after a stressor is applied to the fish, it has been reported that after 24 h, levels of plasma lactate decrease to levels comparable or even lower than those seen in control fish (Simontacchi et al. 2008). In our experiment, we studied the stress response of juvenile Atlantic cod 48 h after IP-injections of LPS, so it is possible that the levels of lactate had already subsided in the LPS-injected fish.

On the other hand, we did not see a significant effect of LPS injection on the levels of total serum cortisol, although a trend towards increased levels was obvious. It is likely that this trend in total cortisol levels was in fact caused by the action of the injection per se, as the levels of the saline injected fish were increased to levels comparable to those of the LPS-injected fish (see Fig. 1).

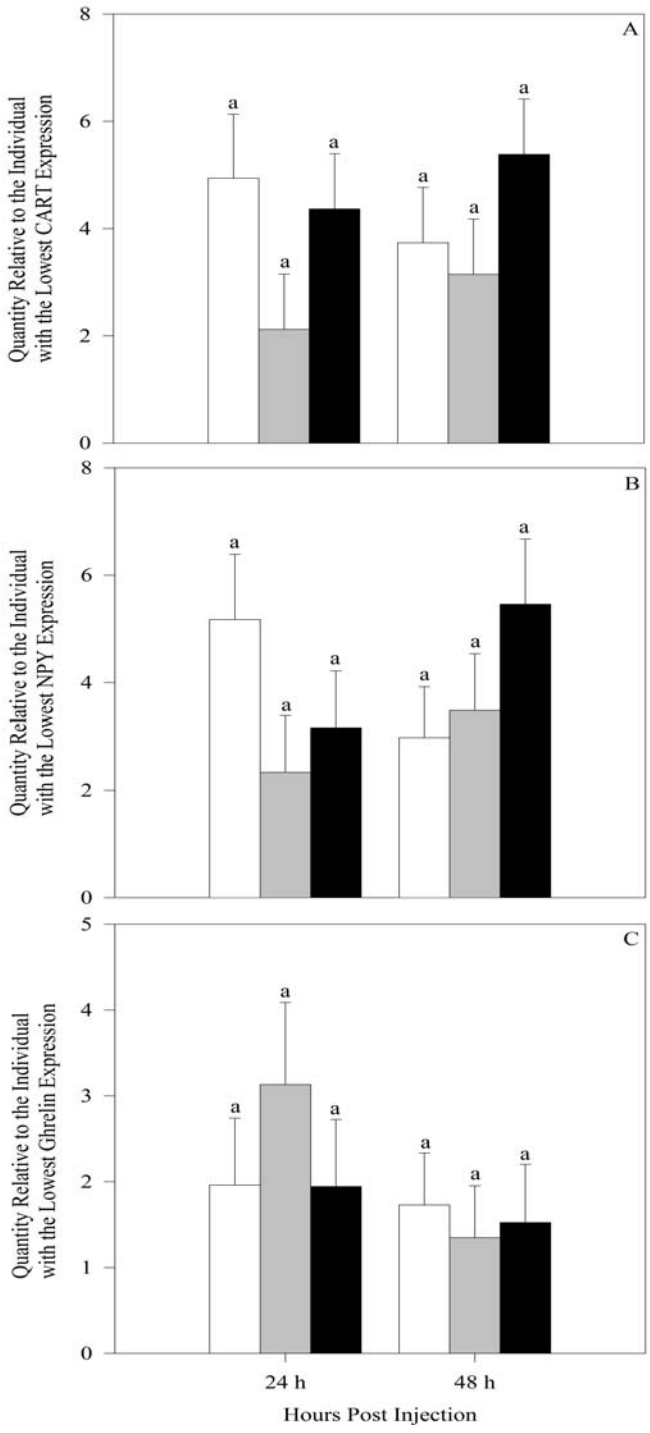

Figure 2. QPCR analysis of CART (A) and NPY (B) mRNA expression in brain and of ghrelin (C) mRNA expression in gut of juvenile cod 24 h and 48 h after injection. Two way ANOVAs were used to analyze each gene. White bars represent the control group, gray bars the saline-injected group and black bars the LPS-injected group. Values are means ± st. dev. Similar letters denote lack of statistical difference ($P > 0.05$; $n = 4$).

To investigate the possible effects of LPS on feeding, we measured the expression of brain and gut mRNA of two orexigenic peptides, NPY and ghrelin, and one anorexigenic peptide, CART. Our results indicate that a single IP injection of 0.5 ng g^{-1} of LPS does not significantly affect the expression of these peptides. This was somewhat expected as feeding levels were similar in control, saline-injected and LPS-injected fish (data not shown) 24 h and 48 h after injection. However, our results are in contrast with studies in goldfish showing that LPS treatment (100 ng g^{-1} of LPS from Escherichia coli) decreases food intake and significantly decreases expression of NPY mRNA and increases expression of CCK, CRF and CART mRNAs 4 h post-injection (Volkoff and Peter 2004). In our study, we IP-injected juvenile Atlantic cod with a dose of 5 ng g^{-1}, thus, it is possible that this dose was not high enough to affect feeding and the expression of appetite-regulating peptides.

In summary, our results indicate that exposing juvenile Atlantic cod to IP-injections of LPS from *A. salmonicida* may have a negative effect on their innate immune response as indicated by the decrease of RB of whole blood and head kidney. At doses of 5 ng g^{-1}, LPS did not affect the expression of appetite regulating factors or the physiological stress response of Atlantic cod. Our results provide scientists who may want to use LPS injection to mimic infection outbreaks with some information on potential reactions of Atlantic cod to LPS. Further studies (e.g., higher doses) are necessary to better understand the impact that LPS may have on the regulation of food intake and immune responses in fish.

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