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Potential molecular biomarkers of crowding stress in Atlantic cod, *Gadus morhua* and their importance in health management

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Abstract. Sub-adult Atlantic cod, *Gadus morhua* were subjected to short-term crowding stress in order to map out potential biomarkers of stress response of this fish at the molecular level. This short review summarizes the results of the different studies done to better understand the stress physiology of this fish. Short-term overcrowding resulted in the increase in expression levels of genes related to antibacterial activity (BPI/LBP and g-type lysozyme), inflammation (IL-1β and IL-8), metabolism and antioxidant defense. It also demonstrated that most of the gene transcripts attained peak expression levels even when the plasma cortisol had returned to its baseline level. The expression of GLUT-3 and -4 significantly increased as a result of overcrowding, indicating the possible roles of these genes in the stress response particularly during glucose metabolism. Among the antioxidants, the Cu/Zn SOD maintained high expression levels, thus, can be potentially used a molecular biomarker for crowding stress.
Key Words: Atlantic cod, *Gadus morhua*, biomarker, stress.

Introduction. The stress response is a vital aspect of the physiological make-up of living organisms. Similar to other vertebrates, a fish will undergo a series of biochemical and physiological changes in order to compensate for the challenges imposed on it (Wendelaar Bonga 1997). The mechanisms how fish responds to stressors have been studied in great detail because stress has an apparent effect on the welfare and productivity of aquacultured fish. Various stressors such as grading, transporting, crowding and vaccinating are inevitable especially in modern aquaculture. Among these stressors, crowding due to increased stocking density is considered as an aquaculture-related chronic stressor, which produces a chronic elevation of plasma cortisol (Mommsen et al 1999). Plasma cortisol is generally used as an indicator of stressful situations in vertebrates including fish, and when there is an elevation in its level, it induces a series of secondary physiological changes in the organism (Wendelaar Bonga 1997; Barton 2002) such as decreased immune competence, increased disease susceptibility and decreased growth and survival rates.

Aside from plasma cortisol, which is used as an indicator of stress levels in fish and other vertebrates, there are other biomarkers of stress conditions in an organism. A biomarker is any biological response, *i.e.*, molecular, cellular and physiological response, to a stress factor measured in an organism indicating a deviation from the normal state. The significance of plasma cortisol, together with other blood parameters such as hematocrit, blood glucose and total haemoglobin, in assessing stress conditions in fish might be limited especially when the responses of chronically stressed fish reared at high stocking densities is concerned (Van Weerd & Komen 1998). This suggests that there are other potential biomarkers of stress response, particularly molecular biomarkers that can be used to augment the information derived from plasma cortisol when monitoring stress response in fish.

Molecular biomarkers reflect gene activity hence they are useful as early indicators when fish undergo stress, and monitoring the transcription levels of single genes can

provide significant information on our understanding of the mechanisms underlying the physiology of the stress response in fish. A comprehensive knowledge of important biomarkers of stress in fish using transcriptome analysis would result in the design of effective husbandry procedures in alleviating stress problems associated with increased stocking density.

A protocol for short-term crowding in fish using Atlantic cod, *Gadus morhua*, as a model for cold-water fish species in the study of the stress response was developed (Caipang et al 2008a, 2008b). In recent years, interest in the farming of this fish has been growing, especially in countries from the colder parts of the Northern hemisphere and it is predicted that the production of farmed cod will reach a similar level as that of Atlantic salmon, *Salmo salar* within the next 15–20 years (Rosenlund 2006), and the largest growth is predicted to take place in Norway (Kjesbu et al 2006). As intensification in the farming of this fish is expected to take place, the fish are more prone to stress due to increasing densities, thereby making them more susceptible to disease-causing pathogens. It is therefore imperative that we obtain a good knowledge on the physiology of the stress as this represents a key parameter in maintaining standards of proper fish husbandry and welfare and improved health status.

Molecular biomarkers of crowding stress. The short-term crowding stress protocol in Atlantic cod involved lowering the water level in the tanks thereby increasing the density of the fish in the tank from 8-10 kg m^{-3} to 100 kg m^{-3} and keeping the fish remained at this density for 1 h (Caipang et al 2008a). The fish were subjected to this pattern of overcrowding thrice over a 12 h interval period. After each exposure time, the water level in the tanks was increased gradually to the original level to facilitate recovery of the fish. Blood samples were collected from the fish before lowering the water level (initial) and at 2, 24 and 72 h post-crowding. Transcription levels of the genes related to antibacterial activity: bactericidal permeability-increasing protein/lipopolysaccharide-binding protein (BPI/LBP) and g-type lysozyme; inflammation: interleukin-1β (IL-1β) and IL-8; metabolism: glucose transporter-1 (GLUT-1), GLUT-2, GLUT-3, GLUT-4 and glycerol-3-phosphate dehydrogenase (G3PDH); and antioxidant defense: catalase, phospholipid hydroperoxide glutathione peroxidase (GSH-Px) and Cu/Zn superoxide dismutase (Cu/Zn SOD), were monitored in order to determine which among these genes are likely candidates as biomarkers of crowding stress. The results of our studies are indicated in Table 1.

Table 1

Expression levels of selected genes in the blood of Atlantic cod following short-term crowding stress

Gene	*Hours post-crowding*			
	Initial	*2 H*	*24 H*	*72 H*
BPI/LBP	+	++	+++	++
g-type lysozyme	+	++	++	+++
IL-1β	+	+++	++	++
IL-8	+	++	++	+++
GLUT-1	+	+	+	+
GLUT-2	+	+	+	+
GLUT-3	+	++	++	+++
GLUT-4	+	+++	++	++
G3PDH	+	+++	++	+
Catalase	+	+++	++	+
GSH-Px	+	+++	+	+
Cu/Zn SOD	+	+++	++	++

+, constitutive expression; ++, significant upregulation; +++ peak expression level. Semi-quantitative measurements of gene expression were obtained from n=14. Adapted from Caipang et al (2008a, 2008b).

The upregulation of antibacterial genes, BPI/LBP and the g-type lysozyme likely indicates that the immune response in Atlantic cod was enhanced following exposure to overcrowding. We believe that the stressor that we applied was an acute one because just like in rainbow trout (*Oncorhynchus mykiss*), mild acute stress resulted in the stimulation of the natural immune system (Demers & Bayne 1997). It is possible that mild acute stress in Atlantic cod is a good modulator of immune response, which might lead to better protection in fish against trauma.

It has been shown that the HPI-axis and cytokines have a bi-directional communication (Engelsma et al 2003). IL-1β is one of the pro-inflammatory cytokines that has a crucial role in mediating several responses after an injury or infection (Engelsma et al 2002). It primes the leukocytes for respiratory burst and phagocytosis upon exposure to pathogens (Secombes et al 2001) and augments the release of another cytokine, IL-8 (Fast et al 2007). Both IL-1β and IL-8 were significantly upregulated following crowding stress and such levels were maintained even at 72 h thereafter. This indicates that an inflammatory response takes place in Atlantic cod when subjected to overcrowding, which may persist even after plasma cortisol has returned to its basal level.

Genes related to glucose transport (GLUT) mediate glucose movement across membranes, and in fish their functions are related to providing glucose reserves during acute or chronic low oxygen challenges (Hall et al 2005). Hyperglycemia, which is a stress response, takes place due to the release of catecholamines (Andersen et al 1991) and is needed to augment the energy demands of the fish for swimming and continuous social adaptation. The levels of GLUT-1 and -2 transcripts in the blood remained relatively unchanged as a consequence of overcrowding suggesting that these genes may not have crucial roles in glucose transport. On the other hand, GLUT-3 and -4 were upregulated, indicating their possible involvement in the stress response of this fish species by serving its metabolic demands. The increased transcription of GLUT-3 and -4 in the blood as early as 2 h post-crowding, indicates that there is a need for enhanced glucose transport to different cells and tissues of the fish in order to meet the increasing energy demands as a consequence of crowding stress. Even if plasma cortisol returned back to its normal levels at 24 h post-crowding, GLUT-3 and -4 transcripts were still highly upregulated. It is likely that there is no need for sustained high cortisol levels in the blood to ensure transcription of glucose transporters to meet energy requirements of the fish as a consequence of crowding stress. Rather, the sudden increase of cortisol in the blood may resulted in the activation of receptors involved in glucose metabolism thereby leading to high glucose production and transport even if cortisol had returned to its basal level in the blood. G3PDH is a key enzyme in freeze resistance among salmonids (Vanya Ewart et al 2001). The enhanced expression of G3PDH in the blood of Atlantic cod post-crowding also suggests the possible role of this metabolic enzyme in the stress response.

All the three gene transcripts related to antioxidant defense in Atlantic cod were significantly upregulated following exposure to crowding. Peak level of expression was observed as early as 2 h after crowding, indicating an immediate response of the antioxidant system in this fish species to protect the cells and tissues from the production of oxygen radicals. Our study showed that the antioxidant system may have critical roles in maintaining cellular metabolism in the fish following exposure to overcrowding. It appears that individual antioxidant enzymes are involved in the mechanism protecting the fish as a consequence of overcrowding, based on the established functions of each antioxidant gene. Cu/Zn SOD detoxifies superoxide anions (O_2^-), catalase is involved in the reduction of hydrogen peroxide (H_2O_2), and GSH-Px reduces both H_2O_2 and organic peroxides.

Among the antioxidant genes, the expression of Cu/Zn SOD remained at significantly higher levels, which persisted even at 72 h post-crowding. The other two antioxidants returned to their basal levels of expression during the 72 h time period. For comparison on the effects of other stressors in Atlantic cod, the GSH-Px and Cu/Zn SOD transcripts in liver were downregulated following hypoxia and upregulated during hyperoxia, whereas catalase remained unchanged when fish were exposed to suboptimal levels of oxygen (Olsvik et al 2006). As more gene sequences are available to enable

transcription analysis in addition to protein assays on the effects of a variety of stressors on fish, the use of individual genes can be useful biomarkers of stress in animals (Bustin 2002).

The study indicates the potential use of Cu/Zn SOD as candidate molecular biomarker of oxidative stress in the blood of Atlantic cod subjected to overcrowding. This is in contrast from Olsvik et al (2006), in which they suggested that GSH-Px might be a useful biomarker for hypoxia and hyperoxia stress in the liver of the same fish species. Hence, the use of biomarkers for evaluation of stress response in fish can be both tissue- and stress-specific. Despite of these differences, these studies clearly show the involvement of the antioxidant defenses in Atlantic cod when they are exposed to wide array of stressors.

Conclusions. The different studies have shown that exposure to short-term overcrowding in Atlantic cod results in the increase in expression levels of genes related to antibacterial activity (BPI/LBP and g-type lysozyme), inflammation (IL-1β and IL-8), metabolism and antioxidant defense. It also revealed that most of the gene transcripts attained peak expression levels even when the plasma cortisol had returned to its baseline level. The expression of GLUT-3 and -4 significantly increased as a result of overcrowding, suggesting that these genes have important roles in the stress response particularly in glucose metabolism. Among the antioxidants, the Cu/Zn SOD maintained high expression levels, which lasted up to 72 h after exposure to crowding and this can be potentially used a molecular biomarker for crowding stress. Further studies on the effects of long-term overcrowding in relation to physiological responses and expression analysis using a larger profile of genes are needed in order to elucidate the mechanisms of the stress response in Atlantic cod and their health status.

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