In vitro effect of acute hypoxia on blood cell metabolism and respiratory burst response in three aquaculture finfish species, cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), and steelhead trout (*Oncorhynchus mykiss*)

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IN VITRO EFFECT OF ACUTE HYPOXIA ON BLOOD CELL METABOLISM AND RESPIRATOY BURST RESPONSE IN THREE AQUACULTURE FINFISH SPECIES, COD (*GADUS MORHUA*), ATLANTIC SALMON (*SALMO SALAR*), AND STEELHEAD TROUT (*ONCORHYNCHUS MYKISS*)

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ABSTRACT

Hamoutene D., Burt K., Samuelson S., Mabrouk G., Mansour A., and Williams, K. 2009. *In vitro* effect of acute hypoxia on blood cell metabolism and respiratory burst response in three aquaculture finfish species, cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), and steelhead trout (*Oncorhynchus mykiss*). Can. Tech. Rep. Fish. Aquat. Sci. 2831: 10 p.

Investigation was conducted on *in vitro* effect of hypoxia on blood cells of three aquaculture finfish species widespread in Atlantic Canada: cod, steelhead trout (anadromous rainbow trout), and salmon. Exposure to 3 hours of hypoxia had no significant effect on antioxidant enzymes, citrate synthase, and lactate dehydrogenase of red blood cells. Similarly, no differences were found when comparing data in red blood cells from the 3 species. This result remains surprising considering known differences in tolerance to hypoxia among these species. The lack of effect on red blood cell activities could be explained by the short-term exposure not allowing a significant change in metabolism to occur. Respiratory burst (RB) activity of steelhead trout and salmon blood was unaffected by hypoxic conditions while cod white blood cells RB was decreased by exposure to hypoxia. Our results suggest a higher sensitivity of cod immune cells to hypoxia. Previous studies on cod immunity have revealed an inferior mechanism in antibody generation compared to other fish species rendering the direct response of immune cells to a stress/pathogen of primary importance in setting up a defense mechanism.

RÉSUMÉ

Hamoutene D., Burt K., Samuelson S., Mabrouk G., Mansour A., and Williams, K. 2009. *In vitro* effect of acute hypoxia on blood cell metabolism and respiratory burst response in three aquaculture finfish species, cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), and steelhead trout (*Oncorhynchus mykiss*). Can. Tech. Rep. Fish. Aquat. Sci. 2831: 10 p.

Des essais *in vitro* ont été effectués pour comprendre l'effet de l'hypoxie sur les cellules sanguines de 3 espèces de poissons aquacoles de l'Atlantique Nord : morue, truite argentée (steelhead trout), et saumon. Trois heures d'hypoxie *in vitro* n'ont eu aucun effet significatif sur les activités d'une enzyme anti-oxydante, de la citrate synthéase, ainsi que de la lactate déshydrogénase des globules rouges. De même, aucune différence entre les espèces considérées ne fut observée. Ce résultat reste surprenant étant donné que les trois espèces considérées sont connues pour leurs tolérances diverses à l'hypoxie. L'absence d'effet sur les globules rouges pourrait peut être s'expliquer par la courte durée d'exposition à l'hypoxie. L'hypoxie, ne semble pas affecter l'activité des cellules immunitaires (mesurée comme la production de radicaux libres) chez la truite et le saumon mais provoque une diminution d'activité chez la morue. Nos résultats suggèrent une sensibilité plus grande des cellules immunitaires de la morue à l'hypoxie. Les résultats d'études effectuées sur les morues ont démontré que ces dernières pourraient posséder un mécanisme inferieur à ceux des autres poissons pour la production d'anticorps. Les réponses directes des cellules au stress/hypoxie seraient donc le mécanisme essentiel de défense immunitaire.

INTRODUCTION

Phagocytosing leucocytes of fish constitute the first line of defence against invading microbial pathogens (Secombes 1996). Studies on respiratory burst (RB) response of fish phagocytes have been widely used to assess the antimicrobial activity (Nikoskelainen et al. 2006). The RB includes reduction of oxygen to water in a step-wise process involving intermediate reduction products like superoxide anion, hydrogen peroxide, hydroxyl radicals and the electronically excited singlet oxygen. All of these forms are implicated in the antimicrobial activity of activated macrophages (Andrew et al. 1985).

Previous studies have shown that *in vitro* exposure of trout erythrocytes to hypoxic conditions results in at least two significant alterations in the catecholamine signal transduction system (Reid et al. 1993), suggesting a direct effect on blood cells. Research conducted on commercial invertebrates demonstrated a decrease in reactive oxygen species (ROS) production in hemocytes of shrimps (LeMoullac 1998) and lobsters (Moss and Allam 2006) subjected to hypoxia. Authors concluded that the lowering of the ROS production could hamper the animal's ability to mount an effective defense against invading microorganisms in areas affected with hypoxia (Moss and Allam 2006).

Resistance to infection and disease can be an important trait for selectively bred aquaculture lines. Any factor influencing immune responses could impact aquaculture production. In the marine cage environment, levels of dissolved oxygen (DO) are often unpredictable, as they depend on other environmental factors, such as light, tidal current, and wind for water flow and oxygen mixing (e.g. Braithwaite and McEvoy 2005). In this study, investigation was conducted on *in vitro* effect of hypoxia on blood cells of three aquaculture finfish species widespread in Atlantic Canada. Based on previous data collected in salmon aquaculture cages located in Newfoundland (Mansour et al. 2008), most of the hypoxia episodes consisted of intermittent hypoxic events (i.e. DO <6 mg/L), with a mean duration of approximately 2 hours. Exposure of blood cells to 3 hours of acute hypoxia was followed by evaluation of the antioxidant responses of red blood cells as well as lactate dehydrogenase (anaerobic metabolism) and citrate synthase activity, an enzyme involved in the tricarboxylic acid cycle and a marker for mitochondrial volume (Phillips et al. 2000). Effect of hypoxia on respiratory burst responses of all blood cells and white blood cells in particular was investigated. The physiological response of fish is variable at set levels of water oxygen pressure and different species exhibit varying degrees of tolerance to hypoxia (e.g. Burton and Heath 1980; Pichavant et al. 2002). It has been shown that Atlantic cod is relatively tolerant to hypoxia (Plante et al. 1998). Similarly, Gamperl et al. (1998) have showed that *Salmo salar*'s heart is well adapted for sustained performance under hypoxic conditions. To the contrary, *Oncorhynchus mykiss* is generally considered to be a hypoxia sensitive species (Arthur et al. 1992). This study will contribute to bringing some information on

that matter by comparing blood cell responses to hypoxia in these 3 species with a focus on immune cell innate responses.

MATERIALS AND METHODS

BLOOD COLLECTION AND PREPARATION

Three to four mL of blood was sampled in 2007 and 2008 from Atlantic cod (200-600 g), salmon (150-300 g), or steelhead trout (200-600 g) (fish were acquired from Natures Sea Farm Inc., Newfoundland). Blood samples were placed on ice prior to experimentation. Experiments were conducted on 7 fish and repeated once (for salmon, exposure was conducted only once). 3 mL of blood were diluted in 60 mL of isolation buffer (MCHBSS + Alsever's buffer) (Crippen et al. 2001) and subjected to various conditions of oxygenation (normoxia, hypoxia). As all dilutions were performed in the same buffer, preliminary tests were performed on cod and steelhead trout blood cells to ensure no effect of buffer on cell viability (through lysis or osmotic shock). No effect of buffer used for dilution was found. The diluted blood was maintained in 25 ml glass flasks at room temperature and gassed continuously for 3 hours with a humidified gas mixture. The gas mixtures were purchased already mixed as follows; normoxia: 20% O_2, 79.8% N_2, 0.2% CO_2; hypoxia: 2% O_2, 97.8% N_2, 0.2% CO_2 (Reid et al. 1993; Wood and Simmons, 1994). The gas flow was maintained at a high rate to ensure bubbling and mixing of the diluted blood.

After 3 hours of constant bubbling, blood cells were separated using a different protocol for cod than for salmon and trout as described in the literature (Crippen et al. 2001; Stenvik et al. 2004) and tested in our laboratory (using Crippen's protocol for cod provoked the bursting of cod red blood cells). Protocols were pre-tested on blood cells, cell viability checks using Trypan Blue were conducted, as well as proper separation of cells verified by using ocular microscopy. Cod diluted blood was added to a Percoll gradient 33/51% in PBS and NaCl 1.8% centrifuged at 400xg for 30 minutes according to Stenvik et al. (2004). Diluted salmon or trout blood was overlaid on an 8 mL cushion of Histopaque-1077 following a protocol described by Crippen et al. (2001) and centrifuged at 400xg for 40 min at 4ºC. White blood cells at the interface were harvested while red blood cells formed a pellet that was redissolved in 20 mM Hepes, 1 mM EDTA, 0.1% Triton pH=7 (Phillips et al. 2000). White blood cells were used directly for RB response evaluation, and red blood cells were kept and frozen at -65C prior to enzymatic analysis.

ENZYMATIC ANALYSES

Enzymatic analyses were conducted on red blood cells extracts. Catalase (CA) activity was measured according to the procedure originally devised by

Chance and Herbert (1950). Lactate dehydrogenase (LDH) activity was evaluated as described in Mitchell et al. (1980). Citrate synthase (CS) and superoxide dismutase (SOD) activities were measured using commercial kits, SIGMA CS0720 and SIGMA 19160 respectively. Similarly, Glutathione (GSH) amounts were evaluated using SIGMA kit CS0260. Protein analysis was conducted according to Lowry et al. (1951). CS, SOD, and GSH levels were not measured on cod blood samples.

RESPIRATORY BURST RESPONSES

Oxidative burst was quantified using a flow cytometer (Electron microscopy unit, Health Sciences, St John's) as a measure of intracellular hydrogen peroxide production following activation with Phorbol Myristate Acetate (PMA 1 µg/mL, final concentration). Flow cytometric assessment of the RB was based on the technique described by Bass et al. (1983). The assay depends upon the cell incorporating 2'–7' dichlorofluorescein diacetate (DCFH-DA), a stable nonfluorescent molecule which is hydrolyzed to DCFH by cytosolic enzymes, then by the action of H_2O_2 (produced in leukocytes stimulated with PMA), DCFH is oxidized to fluorescent dichlorofluorescein (DCF). Briefly, isolated circulating leukocytes were incubated with DCFH-DA (5 µM) at room temperature and fluorescence measurements were done to acquire baseline fluorescence levels. PMA was added and fluorescence was measured immediately (time zero) and 10 min after cell stimulation. Adding PMA provoked only a two-fold increase in fluorescence (in all samples); ''time zero'' levels of fluorescence were considered when calculating stimulation index. Between 10,000 and 30,000 events were included in the analysis of every blood sample (analysis was conducted on whole blood as well as white blood cells only). 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 zero''. Cellular debris were excluded from the analysis by raising the forward-scatter threshold only minimally.

STATISTICAL ANALYSIS

Data for cod and steelhead trout were compared by using two-way ANOVA (α=0.05) with factors being trial (2 trials) and treatment (normoxia and hypoxia). For salmon parameters, a paired t-test was conducted on all data (α=0.05).

RESULTS

ENZYMATIC ANALYSES

It is important to state that all incubations were carried out at room temperature. Therefore, cell viability was checked to ensure no effect of temperature on cod and steelhead trout blood cells (after 3 hour and 24 hours). No effect on cell viability was found after incubating cells at 4ºC or at room temperature with 94% viability for cod cells and 87% viability for steelhead trout cells, both unchanged even after 24 hour (4ºC or at room temperature). No significant differences in enzymatic levels, GSH, or protein amounts were found between red blood extracts of cod, salmon, or trout submitted to normoxic vs. hypoxic conditions (Table 1 and Table 2).

Table 1. Enzymatic activities (CA, SOD, CS, LDH) as well as GSH and PROT data of red blood cells extracts of cod, steelhead trout, and salmon after *in vitro* exposure to normoxic and hypoxic conditions.

	Cod		Steelhead trout		Salmon
	Trial1 (n=6)	Trial2 (n=7)	Trial1 (n=7)	Trial2 (n=7)	Trial1 (n=7)
CA- N	81.8±20.4	140.3±38.7	188.1±72.5	350.8±57.9	305.2±114.0
CA- H	75.1±11.5	100.5±55.5	150.8±65.1	366.5±66.6	323.0±126.1
SOD- N	No data	No data	0.023±0.007	0.026±0.003	0.019±0.005
SOD- H			0.029±0.009	0.023±0.006	0.021±0.005
GSH- N	No data	No data	0.368±0.098	0.385±0.182	0.304±0.084
GSH- H			0.085±0.089	0.327±0.135	0.318±0.133
CS- N	2.87±1.19	5.22±3.30	3.18±1.50	4.27±2.07	3.64±9.27
CS- H	4.00±2.17	6.66±3.91	3.39±2.07	4.34±1.07	4.47±1.18
LDH- N	200.54±49.88	199.13±52.81	32.20±42.49	19.50±4.36	11.45±9.81
LDH- H	93.91±20.26	208.51±51.37	28.67±15.90	24.82±15.19	10.96±2.86
PROT- N	5.21±1.93	6.39±1.94	6.48±1.92	3.25±0.45	4.71±1.20
PROT- H	5.05±1.20	7.02±2.23	4.92±2.36	3.79±0.78	4.45±1.43

CA: catalase (units/mg prot), SOD: superoxide dismutase (units/mg prot.), GSH (nmoles/mg prot), CS: citrate synthase (nmoles/min/mg prot.), LDH: lactate dehydrogenase (nmoles/min/mg prot.), PROT: proteins (mg/ml). N: Normoxia, H: Hypoxia.

Table 2. Results of two-way ANOVAs conducted on enzymatic activities (CA, SOD, CS, LDH) as well as GSH and PROT data of red blood cells extracts of cod and steelhead trout.

	Cod			Steellhead trout		
	Trial effect	Treatment effect	Interaction trial-treatment	Trial effect	Treatment effect	Interaction trial-treatment
CA	No	No	**Yes**	Yes	No	No
SOD				No	No	**Yes**
GSH				**Yes**	**Yes**	**Yes**
CS	**Yes**	No	No	**Yes**	No	No
LDH	**Yes**	No	No	No	No	No
PROT	**Yes**	No	No	**Yes**	No	**Yes**

CA: catalase, SOD: superoxide dismutase, GSH, CS: citrate synthase, LDH: lactate dehydrogenase, PROT: proteins. Yes: significant differences (P<0.05) in one factor (trial or treatment) after allowing for effects of differences due to the other factor. No: no significant differences.

A significant difference in GSH levels due to hypoxia can be observed in steelhead trout, nonetheless, this is to be considered with caution as an interaction between the trial and the treatment can be seen (basically hypoxia could have a different effect on cells depending on the fish/trial). Results mostly indicate that significant differences in enzymatic activities exist between *in vitro* trials for the same species. In general, parameters show no effect of *in vitro* hypoxia on red blood cell metabolism in cod, steelhead trout and salmon.

RESPIRATORY BURST RESPONSES

Ratios of RB stimulation were calculated as described in the material and methods section for whole blood (WB) and white blood cells (WBC) after separation. All results are summarized in Table 3 and Table 4 (ANOVA).

Table 3. Ratios of respiratory burst responses for whole blood and white blood cells after separation.

	Cod		Steelhead trout		Salmon
	Trial1 (n=6)	Trial2 (n=7)	Trial1 (n=7)	Trial2 (n=7)	Trial1 (n=7)
WB- N	6.34±1.04	4.83±1.07	13.53±7.23	3.90±1.25	3.46±0.77
WB- H	6.82±4.27	4.94±1.72	16.86±9.71	6.82±3.67	4.00±1.44
WBC- N	2.86±0.80	5.95±2.04	5.21±1.20	6.07±2.45	3.93±0.95
WBC- H	1.99±0.21	4.95±0.64	5.75±1.25	7.74±3.40	3.79±1.56

Values in bold indicate statistically significant differences. N: normoxia, H: hypoxia.

Table 4. Results of two-way ANOVAs conducted on respiratory burst responses (WB and WBC) of blood cells of cod and steelhead trout.

	Cod			Steellhead trout		
	Trial effect	Treatment effect	Interaction trial-treatment	Trial effect	Treatment effect	Interaction trial-treatment
WB	**Yes**	No	No	**Yes**	No	No
WBC	**Yes**	**Yes**	No	No	No	No

WB: whole blood, WBC: white blood cells. Yes: significant differences (P<0.05) in one factor (trial or treatment) after allowing for effects of differences due to the other factor. No: no significant differences.

ANOVA on stimulation index calculated as ratios of RB show no effect of hypoxia on innate immune responses in trout and salmon cells. A significant decrease in cod WBC respiratory burst can be observed after exposure to hypoxia. Despite differences in the values obtained in the two trials, no interaction can be found between the two factors (trial and treatment) confirming therefore that the effect obtained is similar and independent of the trial considered (different fish, potential differences in temperature, humidity etc.).

DISCUSSION

Respiratory burst by blood cells is a major antimicrobial mechanism in vertebrates and invertebrates. This oxidative process starts when stimulation of macrophages (or other cells) leads to increased consumption of oxygen, the reduction of which, catalyzed mostly by a membrane-bound NADH oxidase, initiates the cascade and production of several reactive oxygen species (Moss and Allam 2006). As all incubations were carried out at room temperature, cell viability testing was set-up to ensure no effect of temperature. Results showed no cell death when data was compared to viability percentages assessed when cell were incubated at 4ºC. It is commonly stated that the innate immunity parameters are relatively temperature independent (Magnadottir 2006). An example of this is the wide temperature range of cod phagocytic activity, which appears to be as active at 4ºC as at 15ºC (Nikoskelainen et al. 2006). No differences were found in RB ratios among species. Nikoskelainen et al. (2006) found that the RB capacity of cod was significantly higher compared to rainbow trout cells or, that the number of blood phagocytes was greater in cod blood. In this study, ratios were calculated after 10 minutes of cell stimulation so differences can still occur after that, moreover, PMA and not zymosan was used as an activator explaining potential differences in cell responses among the 2 studies.

Our results show that the effect (or lack of effect) of hypoxia on red blood cell metabolism demonstrated no differences among species despite the fact that they are known for their varying degrees of tolerance to hypoxia (Plante et al.

1998; Gamperl et al. 1998; Arthur et al. 1992). Side effects of hypoxia include the formation of free radicals causing severe alterations in cellular activities; prevention of such damages is made through defense strategies (small antioxidant molecules, enzymes) (Warner 1994). In any case, subsequent stimulation with a specific trigger of RB could be impaired, or unreliable (Moss and Allam 2006). No consistent effect on antioxidant enzymes has been observed in this study. This could be explained by the short-term exposure not allowing a significant change in metabolism to occur or the fact that, as suggested by Olsvik et al. (2006) in a study on cod, oxidative stress index could be an unsuitable marker for hypoxia since fish can maintain the glutathione system unaltered even under stress. Results of RB show no effect in cell responses in trout and salmon while cod WBC demonstrate a decrease in RB. It has been suggested that acute stress stimulates the natural immune system rather than suppresses it in order to protect fish against possible trauma (Demers and Bayne 1997). Similarly, stimulation of immune responses has been considered to be an indication of lesser toxicity than an inhibition of these same responses (Pipe et al. 1999). Our results suggest a higher sensitivity of cod immune cells to hypoxia. Studies of the genetic mechanism which creates antibody diversity have so far revealed an inferior mechanism in cod compared to other fish species (Stenvik et al. 2000). This particularity would therefore render the reactivity/direct response of immune cells to a stress/pathogen of primary importance in setting up a defense mechanism. It is important to state that the immune response investigated in this study is a direct cell response not mediated by hormones. Considering that the acquired immune response in fish is sluggish compared to the instant and relatively temperature independent innate immune response (Magnadottir 2006), the study of direct cell responses are of great importance for the understanding of innate immunity.

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