Gene Expression in Rainbow Trout (*Oncorhynchus mykiss*), Nile Tilapia (*Oreochromis niloticus*) and White Sturgeon (*Acipenser transmontanus*) Erythrocytes under Differing *in vitro* Glycemic Conditions

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Abstract

Rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*) and white sturgeon (*Acipenser transmontanus*) blood cells were maintained *in vitro* over a 24 h period under different glycemic conditions to compare cellular response to prolonged hyperglycemia. In rainbow trout, facilitated glucose transporter 1 (GLUT 1) expression was upregulated within 1 h regardless of glucose concentration and returned to levels similar to initial quantitative values within 24 h. Likewise, expression of heat shock 70 protein (Hsp 70) was also significantly upregulated after 1 h and remained higher under hyperglycemic conditions compared to normoglycemia until 24 h. Expression of constitutive heat shock protein 90 (Hsp 90) showed significant individual variation but remained unchanged. Expression of GLUT 1 in Nile tilapia remained unchanged under normoglycemic conditions but steadily increased over time with hyperglycemia. Hsp70 and Hsp 90 expression in Nile tilapia was highly variable over time with significant increases observed within 1 h and expression of both genes remaining elevated after 24 h. Significant apoptosis was observed with white sturgeon blood cells under 5 mM and 20 mM glucose *in vitro*. Significantly lower glucose concentrations (0 mM and 2 mM) were required for sturgeon cells to survive. Facilitated glucose transport across membranes via GLUT 1 is responsive to extracellular glucose levels in a variety of fish species, suggesting GLUT 1 may be important in cellular functionality under hyperglycemic conditions.

Keywords: *gene, rainbow trout, tilapia*

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Introduction

The uptake of sugar into red blood cells has been studied and reviewed for several species (Ingermann *et al*. 1984, 1985; Soengas and Moon 1995) and the subsequent metabolism of this substrate has been characterized in rainbow trout (Walsh *et al*. 1990) and other species (Pesquero *et al.* 1992; Tiihonen and Nikinmaa 1991a, 1991b). Erythrocytes in fish are nucleated and able to fully metabolize glucose for energy (Walsh *et al.* 1990). During stressful events, hepatocytes break down glycogen into glucose and release it into the blood stream as part of a response to different toxicological, hormonal, environmental, pharmacological and nutritional conditions (Polakof *et al*. 2012).

In general, the stress response in fish has also been well characterized (Wendelaar Bonga 1997; Iwama *et al*. 2006) as well as a more specific understanding of the stress response regarding heat shock proteins (Iwama *et al.* 1999; Ojima *et al*. 2005) and stress-related hyperglycemia (Momoda *et al.* 2007; Pemmasani *et al*. 2011) in rainbow trout. Previous work has demonstrated increased gene expression of inducible glucose transporter 1 (GLUT 1) and heat shock protein 70 (Hsp 70) in rainbow trout erythrocytes under heat shock stress (Powell *et al.* 2012). We hypothesize that erythrocytes may also recruit increased numbers of glucose transporters to the cell membrane to facilitate cellular uptake with "nutritional stress" associated with hyperglycemia. Although Santin *et al.* (2013) did show glycated hemoglobin to be a poor indicator of plasma glucose levels over time, the nature of increased cellular uptake of glucose by erythrocytes and the subsequent metabolism or binding of glucose under hyperglycemic conditions remains unclear.

In this study we examined the comparative genetic response of rainbow trout, Nile tilapia and white sturgeon blood cells incubated over 24 hours with 5 mM glucose (normoglycemia) or 20 mM glucose (hyperglycemia).

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Materials and methods

Rainbow trout, Nile tilapia, and white sturgeon were raised at Hagerman Fish Culture Experiment Station, University of Idaho, on commercial diets. Rainbow trout (445 ± 23 g) were kept indoors in 140 L tanks at 15 ^{o}C. Tilapia (418 ± 55 g) were kept indoors in 140 L tanks at 26 ^{o}C. Sturgeon (2407 ± 180 g) were kept outdoors in 500 L tanks at 15 ^{o}C. Fish (N = 22) were netted from tanks after fasting for ~24 hours and anesthetized in phosphate-buffered MS-222 (40 mg/ml); 2.5 ml of blood was drawn from the caudal vein, using 22-gauge needles, into 3.0 ml syringes containing 0.5 ml of 5 mM Citrate Phosphate Dextrose Adenine (CPDA; Hillyer *et al*. 2006). Pre-exposure blood samples were removed directly from the syringes and placed into 2 ml microcentrifuge vials containing 1 ml of TRIzol Reagent for RNA extraction. From the remaining blood, 0.5 ml was transferred into each of two dialysis cassettes (Thermo Scientific, Rockford, IL) per fish and marked as either control (5 mM glucose) or challenge (20 mM glucose). Cassettes were submerged into one of two corresponding 2.5 L Pyrex dishes (Model #08-741H, Fisher Scientific, Waltham, MA) with constant stirring and containing 1032 ml Modified Cortland's salt solution (Houston *et al*. 1985) and 168 ml CPDA with either 5 mM or 20 mM added glucose (pH 7.4, 300-340 mmol L^{-1} osmolality). Trout and sturgeon erythrocytes were incubated at 15 ^{o}C, and tilapia erythrocytes were cultured at 26 ^{o}C under sterile conditions. Approximately 100 µl of blood were removed from each cassette at 1, 4, 8, and 24 h post-exposure to the solution, and placed into 2 ml microcentrifuge vials containing 1 ml of TRIzol Reagent for RNA extraction. A point-of-care blood analyzer, VetScan, i-STAT 1 and C 4+ cartridges (Abaxis, Union City, CA) were used to measure pre-exposure blood parameters from separate 0.1 ml samples taken in 1.0 ml syringes during initial sampling.

RNA extraction and quantitative PCR (qPCR) was carried out according to Wayck *et al*. (2012). Gene sequences examined with qPCR included proteins involved in facilitated glucose transport (GLUT 1) and heat shock response (heat shock protein 70: Hsp70 and heat shock protein 90: Hsp90). Primer and probe sequences for ubiquitin (internal standard for trout), glyceraldehyde phosphate dehydrogenase (internal standard for tilapia) and Hsp70 were designed using Primer Express v 3.0 (Applied Biosystems, Foster City, CA).

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Primers for GLUT 1 were from Amberg (2008). Primers for Hsp90 were from Wacyk *et al.* (2012). Quantitative PCR was performed using the AB 7500 Fast Real Time PCR system with the TaqMan Universal RT-PCR Master Mix Reagents Kit (ABI, Foster City, CA). Cycle threshold values were obtained for all genes and linear regression carried out as described in the ABI PRISM User Bulletin #2 (Applied Biosystems, Foster City, CA). Expression was normalized with ubiquitin for trout and glyceraldehyde phosphate dehydrogenase for tilapia. Gene expression was normalized using the absolute relative method (Bustin 2006). Results were analyzed using repeated measures ANOVA in SAS version 9.3 (Cary, NC) and post-hoc SNK tests with a critical value of $\alpha \leq 0.05$.

Blood smear slides were made pre-exposure and 24 hours post-exposure to the modified Cortland's / CPDA solutions. Smears were air dried, fixed in absolute methanol and stained with Stat stain (Volu-Sol, Salt Lake City, UT) as per the manufacturer's instructions. Slides were photographed using a Ziess Axioplan 2 imaging light microscope with mounted AxioCam HRc camera, and individual erythrocytes were qualitatively assessed for membrane characteristics and level of vacuolization then measured for overall area using Axiovision imaging software version 4.7.1 (Carl Zeiss, Göttingen, Germany) (n = 100 cells / time point). Results were analyzed using paired *t*-tests in SAS version 9.3 (Cary, NC).

Results and conclusions

Gene expression patterns of rainbow trout and Nile tilapia overall were markedly different which may reflect the differing dietary regimes of each species. Salmonids such as rainbow trout are carnivorous whereas tilapine fish are omnivorous. Expression of GLUT 1 in rainbow trout (Figure 1a) increased within 1 h regardless of glucose concentration and returned to initial levels within 24 h. However, incubation with 20 mM glucose showed a trend toward increased expression and increased variability in 4 and 8 h samples. Likewise, Hsp 70 expression (Figure 1c) mimicked the GLUT 1 expression pattern but unlike GLUT1, Hsp 70 expression levels at 20 mM glucose were significantly higher at 4 and 8 h compared to expression levels at 5 mM glucose. Hsp 70 expression returned to initial levels within 24 h, a pattern also observed with Hsp 70 response to heat stress (Lewis *et al*. 2010). Hsp 90 expression (Figure 1e) showed elevated expression over initial samples at 8 h at

both 5 mM and 20 mM glucose. Hsp 90 expression diminished to levels consisted with initial values by 24 h.

Expression of GLUT 1 in Nile tilapia (Figure 1b) remained unchanged from initial values over time when blood cells were incubated in 5 mM glucose. Incubation at 20 mM glucose showed increased expression of GLUT 1 beginning at 4 h and remaining elevated after 24 h. Hsp 70 expression in Nile tilapia (Table 1d) varied with generally increasing expression over time regardless of the level of glucose in the culture media. Likewise, Hsp 90 expression (Table 1f) was also observed to increase over time with the highest levels at 24 h.

Table 1. Hematological values and cell sizes for rainbow trout, Nile tilapia and white sturgeon erythrocytes cultured for 24 h at 5 mM and 20 mM glucose.

Parameter	RainbowTrout	Nile Tilapia	White Sturgeon
Hemoglobin (g/dl)	11.6 ± 0.3	6.0 ± 0.2	8.9 ± 0.5
Hematocrit (% PCV)	50.7 ± 1.1	15.7 ± 2.4	29.4 ± 1.3
Initial Glucose Concentration (mM)	3.89 ± 0.11	2.02 ± 0.23	2.52 ± 0.18
Erythrocyte area (µm²)			
Initial	98.06 ± 0.51[a]	62.39 ± 1.64[a]	164.75 ± 3.85
24 hours [5 mM glucose]	107.22 ± 0.56[b]	76.73 ± 1.50[b]	–
24 hours [20 mM glucose]	104.69 ± 1.62[b]	82.59 ± 2.39[c]	–

Hematological values are means of N=6 fish / species ± SEM
Erythrocyte areas are means of N=100 cells / species ± SEM

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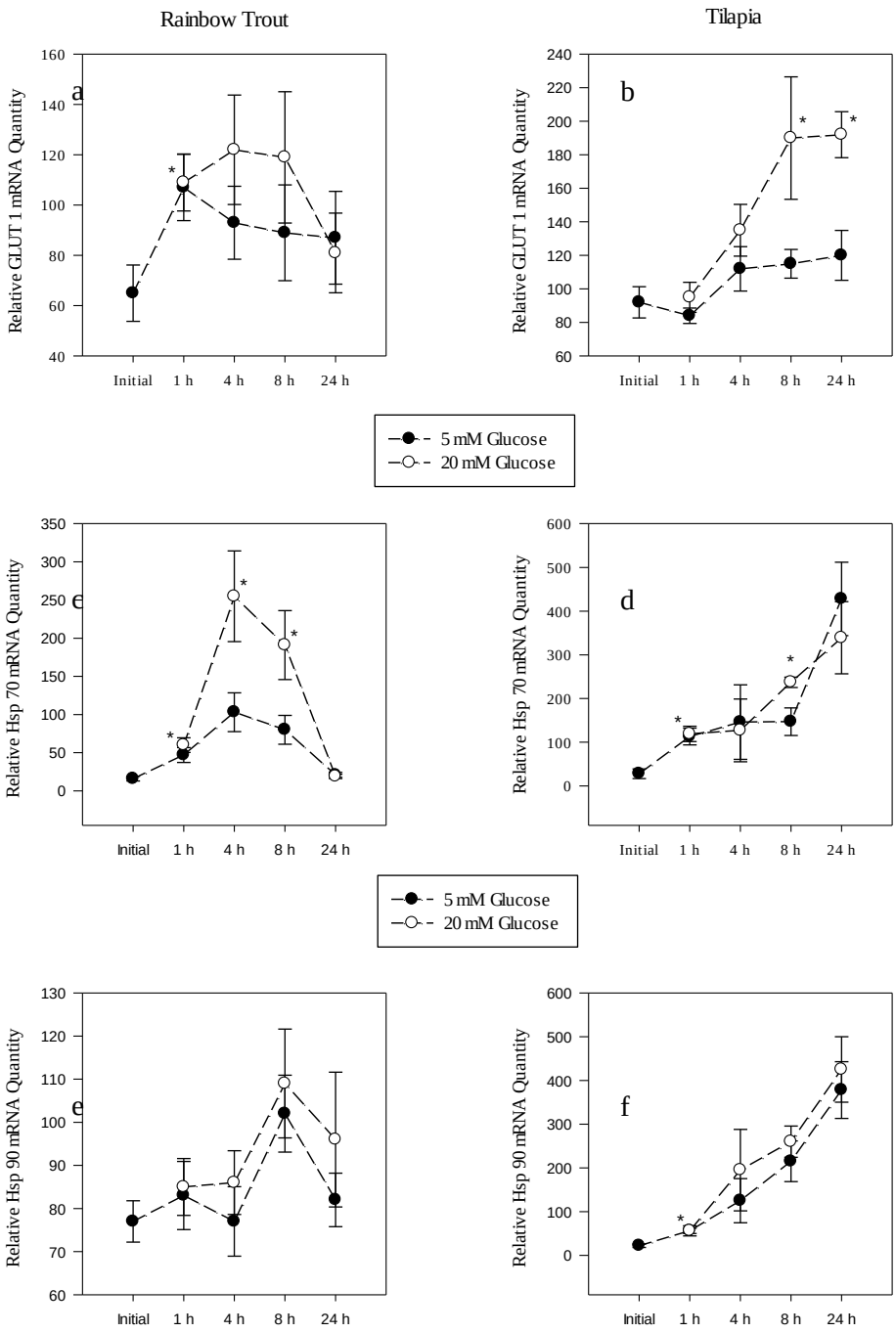

Figure 1. Expression of GLUT 1, Hsp 70 and Hsp 90 genes in rainbow trout and Nile tilapia erythrocytes cultured over 24 h with 5 mM and 20 mM glucose.

Modified Cortland's / CPDA media appeared to be an effective media to incubate rainbow trout blood cells *in vitro*. Cell size of rainbow trout erythrocytes (Table 1) increased by 24 h

from initial measurements but sizes were not significantly different between treatments and did not differ from previously reported cell area measurements (Powell *et al.* 2012). Moreover, quantitative PCR of apoptotic genes (Caspase 3, Caspase 8, Caspase 9) and other stress related genes (superoxide dismutase, thioredoxin reductase, glutathione peroxidase, carbonic anhydrase) did not indicate evidence of stress in rainbow trout blood cells in a previous study (Powell *et al.* 2012). Modified Cortland's / CPDA may be less effective for incubating Nile tilapia blood cells (Table 1) because there was a significant increase in cell size over time between control (5 mM glucose) and challenge (20 mM glucose) treatments. Hematological values (Hg, Hct and plasma glucose) for rainbow trout, Nile tilapia and white sturgeon (Table 1) were within previously reported ranges for these species (Watson *et al*. 1998; Goda 2008; Zhou and Yue 2008; Polakof *et al*. 2012). Although hematocrit and hemoglobin values obtained from the point-of-care blood analyzer were relatively low for tilapia in comparison with previous studies, these values may reflect differences between the sizes of fish used is this study compared to the much smaller fish previously studied. Quantitative examination of apoptotic and stress related gene expression will be useful to further assess the effectiveness of the protocol for incubating Nile tilapia blood cells since the observed increasing Hsp 70 and Hsp 90 expression over time may indicate accumulating cellular stress. Modified Cortland's / CPDA media did not appear to be appropriate for incubation of white sturgeon cells as evidenced by extensive morphological changes and cellular death by 24 h in both treatment and control samples. As a result, further qPCR analysis was not undertaken with the white sturgeon samples but, further analyses should be undertaken using media with differing (lower) glucose concentrations and differing osmolalities.

The similar increases in expression of GLUT 1 over time in response to higher concentrations of extracellular glucose in rainbow trout and Nile tilapia cultured blood cells (albeit responses differing in timing and duration) suggest indirect evidence that GLUT 1 in fish also belongs to the glucose-regulated protein family of stress-induced proteins as observed in mammals (Wertheimer *et al.* 1991). The differences in GLUT 1 expression between rainbow trout and Nile tilapia erythrocytes may be a consequence of different control mechanisms within the cell or the lack of extracellular control (*e.g.* lack of circulating hormones etc.) in the culture system. Although the uptake and use of glucose in

erythrocytes have been examined in several species including rainbow trout, sea raven and American eel (Ferguson and Storey 1991; Sephton and Driedzic 1994; Soengas and Moon 1995), less is known about tilapia red cell uptake and metabolism.

In terms of nutrition or diet formulation for these aquaculture species, rainbow trout erythrocytes appeared to attenuate their response to elevated extracellular glucose within 24 h and culture conditions notwithstanding, tilapia erythrocytes did not. What this says about utilization of dietary glucose or carbohydrates is difficult to decipher. More than likely GLUT 1 plays an important role in erythrocyte functionality during periods of hyperglycemia whether it is induced by diet or acute/cumulative stress. What overarching role this plays in primary or secondary stress responses in erythrocytes as well as any fundamental differences between rainbow trout and Nile tilapia will require further examination of functional genomic pathways.

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