

HORMONAL REGULATION OF GLUCOSE KINETICS IN RAINBOW TROUT: EFFECTS OF INSULIN AND GLUCAGON

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SUMMARY

Mammals and fish rely on hormones to regulate blood glucose levels. The two major glucose regulating hormones are insulin and glucagon. Literature on mammalian insulin and glucagon is quite extensive, however, there is limited information on how these hormones regulate blood glucose levels in fish. The material available for fish mostly pertains to changes in glucose concentration and gene expression of enzymes, but there is no information on the direct influence they have on glucose kinetics. Therefore, the main goal of my thesis is to measure the change in hepatic glucose production and glucose disposal when rainbow trout are administered insulin or glucagon.

The beginning of my research focused on insulin. I hypothesized that rainbow trout respond to insulin by decreasing hepatic glucose production and increase glucose disposal, just like mammals. To test this, I infused insulin for 4 hours at $1.5 \mu\text{g insulin kg}^{-1} \text{ min}^{-1}$. I measured glucose disposal (R_d glucose), hepatic glucose production (R_a glucose), and blood glucose concentration. Following insulin administration the glucose fluxes decreased steadily (R_d glucose -37% and R_a glucose -43%). The decline in blood glucose levels follows the difference between R_d and R_a . These results explain why rainbow trout are unable to clear a glucose load to the same degree as mammals.

The second major glucose hormone (glucagon) is what interested me for the second part of the research. The limited information on fish glucagon is even less than

that of fish insulin. I speculated that trout respond to glucagon the same way mammals do (increase hepatic glucose production and show no affect on glucose disposal). To study the effects of glucagon on glucose fluxes, I tracked the changes in R_a and R_d glucose. The results showed glucose fluxes showed no significant difference from baseline in the first few hours, then steadily decreasing until the final time point reached values below baseline. Therefore, these experiments revealed that glucagon follows a similar pattern of effects in trout as mammals. However, the strength of the response to glucagon is different between trout and mammals.

This thesis is the first to investigate the effects of insulin and glucagon on glucose kinetics in rainbow trout. I have concluded that rainbow trout have different responses to insulin and glucagon when compared to mammals. Furthermore, fish showing limited glucoregulatory capacity can be partially explained by their responses to insulin and glucagon.

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TABLE OF CONTENTS

SUMMARY.....	iii
ACKNOWLEDGMENTS.....	v
LIST OF FIGURES.....	viii
LIST OF TABLES.....	ix
CHAPTER 1 – GENERAL INTRODUCTION.....	1
Introduction.....	2
<i>Metabolic fuels.....</i>	<i>2</i>
<i>Importance of glucose as a metabolic fuel.....</i>	<i>3</i>
<i>Comparing glucoregulators.....</i>	<i>3</i>
<i>Glucose kinetic theory.....</i>	<i>4</i>
Hormones.....	6
<i>Glucoregulatory Hormones.....</i>	<i>6</i>
<i>Insulin.....</i>	<i>6</i>
<i>Glucagon.....</i>	<i>8</i>
Goals.....	11
CHAPTER 2: UNEXPECTED EFFECT OF INSULIN ON GLUCOSE DISPOSAL EXPLAINS GLUCOSE INTOLERANCE IN TROUT.....	15
Introduction.....	16
Methods.....	18

Results.....	23
Discussion.....	25
Conclusions and significance.....	31
CHAPTER 3: REGULATION OF RAINBOW TROUT GLUCOSE FLUXES	
BY GLUCAGON.....	49
Introduction.....	50
Methods.....	52
Results.....	58
Discussion.....	60
Conclusions and significance.....	65
CHAPTER 4: GENERAL CONCLUSIONS.....	89
Thesis overview.....	90
General discussion.....	91
Conclusions.....	95
Future directions.....	95

LIST OF FIGURES

Figure 1.1. Schematic representation of the two compartment model.....	13
Figure 2.1. Experimental design and metabolic rate (MO_2) of resting rainbow trout.....	37
Figure 2.2. Effects of insulin on plasma glucose concentration and glucose specific activity of rainbow trout.....	39
Figure 2.3. Effects of insulin on the glucose fluxes of rainbow trout.....	41
Figure 2.4. Comparative effects of insulin on the glucose fluxes of rainbow trout and humans.....	43
Figure 2.5. Effects of insulin on circulating glucagon levels in rainbow trout.....	45
Figure 2.6. Relative effects of insulin on the levels of key signalling proteins.....	47
Figure 3.1. Effects of glucagon on plasma glucose concentration and glucose specific activity in rainbow trout.....	77
Figure 3.2. Effects of glucagon on the glucose fluxes of rainbow trout.....	79
Figure 3.3. Relative effects of glucagon on muscle mRNA abundance in rainbow trout.....	81
Figure 3.4. Relative effects of glucagon on liver mRNA abundance in rainbow trout..	83
Figure 3.5. Circulating glucagon levels in rainbow trout.....	85
Figure 3.6. Relative effects of glucagon on the levels of key signalling proteins in rainbow trout.....	87
Figure 4.1. Comparative effects of insulin and glucagon on the glucose fluxes of rainbow trout.....	97

LIST OF TABLES

Table 2.1. Mean physical characteristics and hematocrit of the 2 groups of catheterized rainbow trout used (i) for <i>in vivo</i> measurements of glucose kinetics or (ii) for tissue measurements of insulin signalling proteins.....	33
Table 2.2. Initial (baseline) and final values (after 4 h of insulin administration) for various parameters of glucose metabolism and circulating glucagon in rainbow trout.....	35
Table 3.1. Mean physical characteristics and hematocrit of the 2 groups of catheterized rainbow trout used (i) for <i>in vivo</i> measurements of glucose kinetics or (ii) for tissue measurements of glucagon signalling proteins and gene expression of enzymes.....	67
Table 3.2. Initial (baseline) and final values (after 4 h of glucagon administration) for various parameters of glucose metabolism and circulating glucagon in rainbow trout.....	69
Table 3.3. Primer sequences and annealing temperatures.....	71
Table 3.4. Glycogen phosphorylase b transcript numbers in various tissues.....	73
Table 3.5. Glycogen phosphorylase muscle transcript numbers in various tissues...	75

CHAPTER 1
GENERAL INTRODUCTION

Introduction

This thesis investigates the hormonal regulation of glucose fluxes in rainbow trout (*Oncorhynchus mykiss* Walbaum). Rainbow trout are considered glucose intolerant, but recent research shows that they are possibly better gluco regulators than previously thought (Choi and Weber, 2015; Moon, 2001). However, apart from work on epinephrine (Weber and Shanghavi, 2000), there is little information available on the hormones that regulate hepatic glucose production (R_a glucose) and glucose disposal (R_d glucose). Therefore, the aim of this thesis is to investigate the effects of two major glucose-regulating hormones, insulin and glucagon, on glucose kinetics.

Metabolic fuels

All living animals require adenosine triphosphate (ATP), the universal energy currency that supports cellular work for everyday life. Cells have low concentrations of ATP for rapid use but have many different metabolic pathways to match ATP production and ATP utilization (Weber, 2001). Animals obtain energy by consuming food or through body reserves of carbohydrates, lipids and to a lesser extent proteins (Weber, 2011). Proteins are more important for structural and functional roles than energy production because toxic ammonia is produced as a metabolic waste product (Weber, 2001). Lipids make up around 90% of the total energy reserve because they are stored without water; making them light and energy dense. They are only metabolized slowly aerobically which is a drawback (Weber, 2011). Carbohydrates are different from lipids as they are metabolized aerobically and anaerobically, stored in small quantities and can produce ATP at the highest rate compared to other fuels (Weber, 2001).

Importance of glucose as a metabolic fuel

Carbohydrates come in many forms, but the main energy substrate form is glucose. Glucose is accessible from glycogen and/or the circulation, allowing for ATP production. During rest, some tissues rely on glucose as a metabolic fuel. In mammals, the brain accounts for 45-60% of glucose disposal, skeletal muscle 15-20%, kidney 10-15%, blood cells 5-10%, splanchnic organs 3-6% and adipose tissue 2-4% (Shrayyef and Gerich, 2010). The brain relies on glucose more than any other tissue, since the other possible fuels for the brain (ketone bodies and free fatty acids) face transport limitations across the blood-brain barrier (Shrayyef and Gerich, 2010). Inadequate glucose levels can cause serious problems. For example, hyperglycemia can lead to diabetes (retinopathy, renal failure, neuropathy and sexual dysfunction), whereas hypoglycemia can cause damage to the nervous system, coma, and even death. In rainbow trout, indirect approaches to understanding the relative importance of glucose in different physiological situations have been measured. Different environmental, hormonal, nutritional and pharmaceutical conditions change glycemic levels (Polakof et al., 2012). For example, low temperatures and hypoxia lower glucose levels in fish, while stress and hormones like catecholamines, growth hormone, and glucagon increase glucose levels (Polakof et al., 2012). These changes caused by different environmental or hormonal conditions indicate that fish glucose levels are regulated.

Comparing glucoregulators

To ensure proper glucose levels, precise management of glucoregulation is important in mammals and seems to be of some importance for fish. Early studies have relied on glucose tolerance tests to give a simple estimate of the capacity for glucoregulation. In glucose tolerance tests, a known glucose load is administered and the clearance of this glucose load is measured by monitoring changes in blood glucose levels. When mammals (humans, mice and dogs) are given a glucose load of 1 gram glucose/kg body mass (as recommended by the World Health Organization) (Horowitz et al., 1993), they require 1 to 3 hours to restore glycemia to baseline levels (Andrikopoulos et al., 2008; Church, 1980; Horowitz et al., 1993; Kelley et al., 1988; Shrayyef and Gerich, 2010). On the other hand, trout need 24 hours to return to baseline glycemic levels after a 0.25 gram glucose/kg body mass is administered (Legate et al., 2001). These tolerance test results and the seemingly limited sensitivity of fish to insulin (Marín-Juez et al., 2014) have led to the notion, that rainbow trout are poor glucoregulators. Glucose tolerance tests give an idea of an organism's capacity for glucoregulation, but it only measures blood glucose concentration. Changes in blood glucose concentration depend on the balance between the rates of hepatic glucose production and glucose disposal. Glucose concentration only changes when there is a difference between R_a and R_d glucose. Therefore, measurements of blood glucose concentrations only provide limited information on glucose kinetics and glucoregulation.

Glucose kinetic theory

To measure glucose fluxes, the continuous infusion of labelled glucose ([6-³H]glucose) (Haman et al., 1997a; Haman and Weber, 1996) as well as the Steele

steady and nonsteady-state equations (Steele, 1959) will be used. The Steele equations were established by assuming a two-compartment model: the rapidly mixing pool (that includes plasma and extracellular fluid) and a slower-mixing pool made of the intracellular volume of tissues (Fig. 1.1). The tissues are where glucose can be released from or stored in. At rest, the rate of glucose production and glucose disposal are equal to each other resulting in a constant volume for the rapidly mixing pool. When labeled glucose is administered to the rapidly mixing pool, the rate that it is infused at will eventually equal the rate at which it leaves the pool. The animal enters isotopic steady state and the ratio of labeled glucose to unlabeled glucose or specific activity (SA in DPM/ μmol) remains constant. When the infusion rate of labeled glucose (F in DPM $\text{kg}^{-1} \text{min}^{-1}$) and the specific activity are known, we can calculate R_a and R_d glucose ($\mu\text{mol} \text{kg}^{-1} \text{min}^{-1}$) using the steady-state equation,

$$R_a = R_d = \frac{F}{SA}$$

When R_a and R_d are not balanced there is a change in glucose concentration over time. When this mismatch occurs, R_a and R_d can be calculated separately using the nonsteady-state equation as follows,

$$R_a = \frac{F - pV \left(\frac{C_2 + C_1}{2} \right) \left(\frac{SA_2 - SA_1}{t_2 - t_1} \right)}{\left(\frac{SA_2 + SA_1}{2} \right)}$$

$$R_d = R_a - pV \left(\frac{C_2 - C_1}{t_2 - t_1} \right)$$

Where the pool volume is pV ($\text{ml} \text{kg}^{-1}$), the blood glucose concentration (mmol l^{-1}) at time 1 is C_1 and the blood glucose concentration at time 2 is C_2 . The time when blood samples were taken are represented by t_1 and t_2 (min). The pool volume differs between

various metabolites but a glucose pool of 50 ml kg⁻¹ has been shown to yield accurate estimates of glucose fluxes empirically (Haman et al., 1997b).

Hormones

Glucoregulatory hormones

Blood glucose concentration is controlled by the actions of glucoregulatory hormones. These hormones can cause either an increase in blood glucose concentration or decrease it (Polakof et al., 2012; Shrayyef and Gerich, 2010). Trout and mammals use glucagon, catecholamines, growth hormone and cortisol to increase blood glucose levels (Polakof et al., 2012; Shrayyef and Gerich, 2010). Trout have also shown that glucagon-like peptide (GLP) plays a role in increasing glucose concentration (Polakof et al., 2011a) but in mammals GLP enhances insulin secretion and indirectly decreases glucose concentration (Holst, 2007). Furthermore, trout and mammals use insulin and insulin-like growth factor (IGF) to decrease blood glucose concentration (Clemmons, 2004; Polakof et al., 2012; Shrayyef and Gerich, 2010). In trout, the effect these hormones have on glucose fluxes has yet to be measured. Furthermore, the importance of these hormones in glucoregulation has yet to be determined. The two main hormones that ensure tight regulation of blood glucose levels in mammals are insulin and glucagon (Gerich, 1993; Shrayyef and Gerich, 2010). During a glucose tolerance test in mammals the main hormone acting is insulin (Shrayyef and Gerich, 2010) and this may be a similar case in trout (Choi and Weber, 2015).

Insulin

Insulin is highly conserved in many species and is regarded as the major glucoregulatory hormone in vertebrates (Harris et al., 1996; Hernández-Sánchez et al., 2006; Polakof et al., 2012; Shrayyef and Gerich, 2010; Smith, 1966). The active hormone is comprised of an A-chain and a B-chain that are connected by 3 disulfide bonds, and vary in length depending on the species. The hormone is released from the beta cells of the pancreas (mammals) or Brockmann bodies (fish) (Epple, 1969; Shrayyef and Gerich, 2010). Once released, insulin will bind to the highly conserved insulin receptor (IR). The homodimer receptor is comprised of two subunits (the alpha subunit (hormone binding) and the beta subunit (signal transduction) that are linked by a disulfide bond (Caruso and Sheridan, 2011; Saltiel, 2015).

The insulin signalling cascade has been mainly studied in non-piscine species but has major similarities across all vertebrates. The action of insulin begins by the hormone binding to the IR with the B-chain C terminus and the A-chain N terminus (De Meyts, 2004). The IR undergoes conformational changes leading to endogenous tyrosine kinase activation and autophosphorylation of tyrosine residues on the beta subunit. This leads to the movement of the activation loop and opens access for ATP and adaptor proteins (including insulin receptor substrates (IRS), Shc, Gab1, Cbl, and APS) in the cytoplasm to bind via the src homology 2 (SH2) domains (Taha and Klip, 1999). This binding causes changes to their activity and initiates numerous downstream cellular responses through many effector pathways such as PI3K/Akt, ERK, and STAT/Jak-STAT pathways (Youngren, 2007). The major pathway that promotes insulin effects on glucose metabolism, is the PI3K/Akt pathway. PI3K phosphorylates

phosphoinositide (PI) resulting in the binding of secondary messengers PI(3)P, PI(3)P₂ and PI(3)P₃ to PI3K-dependent serine/threonine kinases (PDK1) and Akt. The PIPs recruit Akt and PDK1 to the plasma membrane where Akt undergoes a conformational change, and, is phosphorylated by PDK1. Once Akt becomes phosphorylated (active form), it is able to activate/inactivate components that affect glucose metabolism and cell growth like the major signalling molecule S6 (Caruso and Sheridan, 2011). Previous research shows that varying modes and duration of insulin administration (intraperitoneal bolus (IP) injection (Plagnes-Juan et al., 2008), 11 day-osmotic pump IP infusion (Polakof et al., 2010b), and intravascular (IV) bolus injection (Polakof et al., 2010c)) activates Akt in the liver and muscle. However, Akt and S6 have not been measured after a continuous intravascular infusion for a short time period (4 hours).

In mammals, glucose disposal is tripled from baseline values following insulin infusion (Kelley et al., 1988; Lucidi et al., 2010), but the effect of insulin on glucose disposal has never been measured in fish. When insulin is administered to mammals and trout, the expression of glucose transporters increases allowing the cells to transport glucose across the membrane for processing via glycogen synthesis and glycolysis. In mammals, glycogen reserves accumulate by increasing glycogen synthase activity and strongly inhibiting glycogen phosphorylase, thereby stimulating glycogen synthesis (Saltiel, 2015; Shrayyef and Gerich, 2010). Trout respond to insulin in a similar way. Glycogen abundance increases and glycogen synthase activity is stimulated, but glycogen phosphorylase is unaffected (Polakof et al., 2010c). Insulin affects glycolysis by stimulating the expression of glycolytic enzymes (glucokinase,

hexokinase, phosphofructokinase, and pyruvate kinase) in mammals.

Phosphofructokinase shows no stimulation or inhibition in fish while the status of other enzymes are unclear. Some studies show inhibition of glucokinase, hexokinase and pyruvate kinase expression (Polakof et al., 2010a; Polakof et al., 2010c) and other studies show no response (Enes et al., 2009; Polakof et al., 2010b). Unfortunately, there are no direct measurements of glucose disposal after insulin is administered.

Glucose production is almost completely suppressed following insulin administration in mammals (Kelley et al., 1988; Lucidi et al., 2010), but it is unclear whether fish respond similarly. The liver produces glucose in two ways: glycogen breakdown and gluconeogenesis. Trout and mammals are able to suppress glucose-6-phosphatase expression. However, trout do not inhibit glycogen phosphorylase, dissimilar to mammals who stimulate it (Polakof et al., 2010c; Saltiel and Kahn, 2001). In addition, mammals and trout are able to suppress the expression of major gluconeogenic enzymes (phosphoenolpyruvate carboxykinase, glucose-6-phosphatase and fructose-1,6-bisphosphatase) (Polakof et al., 2010a; Polakof et al., 2010c; Saltiel and Kahn, 2001). Information available suggests that trout may show insulin-driven inhibition of glucose production, but this response has not been measured.

Glucagon

Another highly conserved glucoregulatory hormone in vertebrates is glucagon. Glucagon is a 29 amino acid polypeptide hormone in rainbow trout and many other species (Plisetskaya and Mommsen, 1996). The hormone is released from the alpha

cells of the pancreas (mammals) or Brockmann bodies (fish) (Epple, 1969; Shrayyef and Gerich, 2010). The receptor that binds glucagon in mammals and fish is a part of the G-protein coupled receptor family (Jiang and Zhang, 2003; Plisetskaya and Mommsen, 1996). The receptors in this family share a similar structure of 7 transmembrane helices. The extracellular parts bind hormones while the intracellular parts are connected to G-proteins and transduce the signal inside the cell (Rosenbaum et al., 2009).

The glucagon signalling pathway has been heavily studied in mammals but very little evidence is available on fish. In fish and mammals, glucagon binds to the glucagon receptor (GR) causing a conformational change in the receptor (Moon, 1998). In mammals, the heterotrimeric stimulatory G protein becomes activated by GDP being swapped out for GTP. The $G\alpha$ subunit disassociates from the other two subunits ($G\beta$ and $G\gamma$) (Habegger et al., 2010). $G\alpha$ interacts with adenylyl cyclase or phospholipase C thereby activating the rate limiting enzymes cAMP and inositol-phosphate (IP)/ Ca^{2+} pathways. Although no heterotrimeric G protein has been found in fish. The presence of GTP is able to activate adenylyl cyclase, showing the possibility of G protein presence in trout (Ottolenghi et al., 1988). Mammalian adenylyl cyclase increases cAMP, which in turn, interacts with protein kinase A (PKA) causing responses through multiple cascades. Some of these cascades affect glycogenolysis and others affect gluconeogenesis (Moon, 1998). Furthermore, phospholipase C increases the amount of IP_3 which allows calcium to be released causing activation of the FOXO pathway thereby affecting gluconeogenesis (Habegger et al., 2010). In fish, glucagon is known to

increase cAMP and IP₃ but little information is available about the rest of the signalling cascade (Moon, 1998). Measuring PKA substrates and FOXO1 activation following glucagon infusion has never been performed, and such an experiment would improve current understanding of the glucagon signalling cascade.

The glucose disposal response to glucagon is ambiguous in mammals and has never been measured in fish. Some experiments report that glucose disposal is unaffected by glucagon (Hinshaw et al., 2015; Shrayyef and Gerich, 2010). Other studies describe an inhibitory effect on glucose disposal (Jiang and Zhang, 2003; Lins et al., 1983). Glucagon administered to rat liver and heart decrease glycogen synthesis (Akatsuka et al., 1985; Ciudad et al., 1984; Ramachandran et al., 1983). Further inhibition of glucose disposal is possible in mammals through the decrease in glycolysis via the inhibition of phosphofructokinase-1 and pyruvate kinase expression in rat hepatocytes (Castano et al., 1979; Veneziale et al., 1976). Fish show similar responses to glucagon by decreasing the activity of glycogen synthase (Murat and Plisetskaya, 1977), phosphofructokinase (Foster et al., 1989) and pyruvate kinase (Petersen et al., 1987). However, it is difficult to predict the effect of glucagon on glucose disposal without measuring it directly.

In mammals, glucagon is known to increase glucose production more than glucose disposal, thereby causing a rise in blood glucose levels (Shrayyef and Gerich, 2010). Glucagon is able to stimulate gluconeogenesis and glycogen breakdown in both mammals and fish. Fish respond to glucagon by increasing the expression of

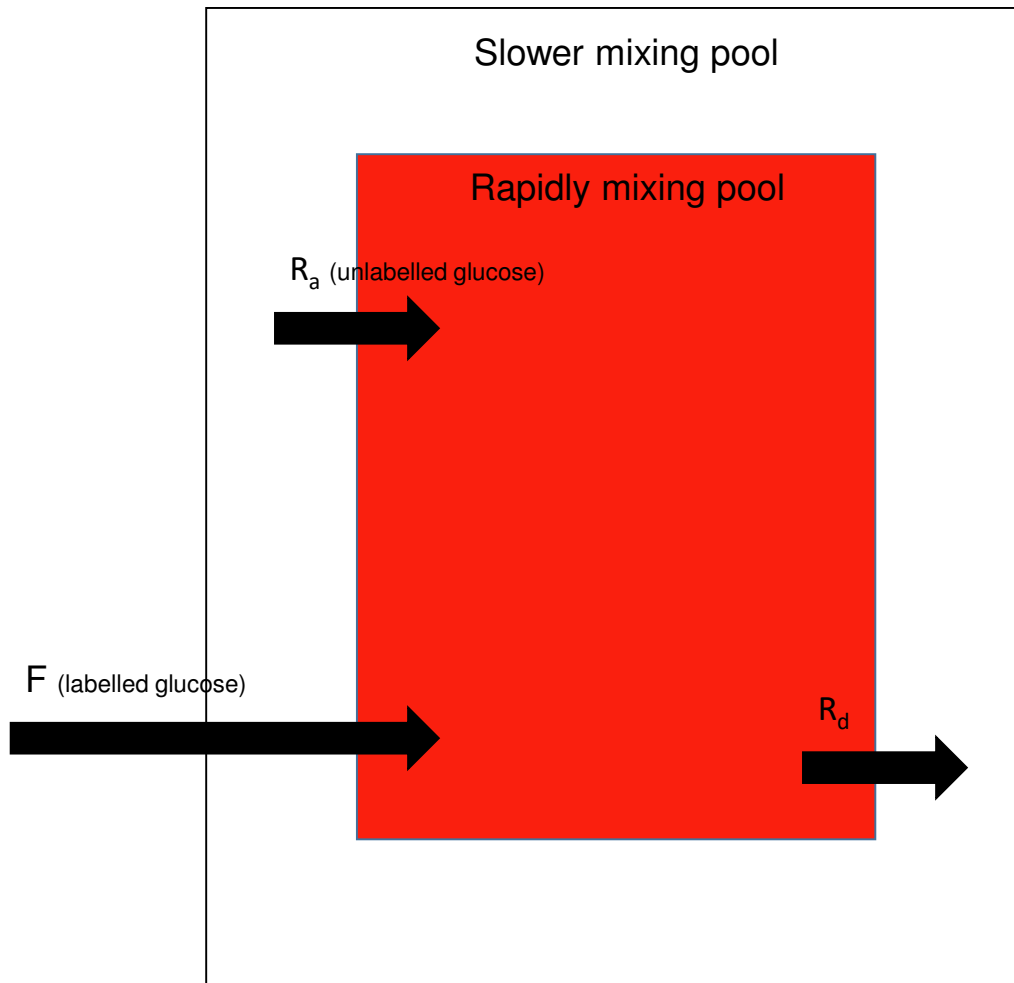
phosphoenolpyruvate carboxykinase (Foster and Moon, 1990a) and increasing the activity of fructose-1,6-bisphosphatase and glucose-6-phosphatase (Sugita et al., 2001): the main enzymes regulating gluconeogenic fluxes. Mammals respond in a similar way by stimulating the expression and activity of these enzymes (Band and Jones, 1980; Beale et al., 1984; Christ et al., 1988; Pilkis et al., 1982; Striffler et al., 1984). Furthermore, glucagon is able to activate glycogen phosphorylase in mammals and fish (Brighenti et al., 1991; Lecavalier et al., 1989; Puviani et al., 1990). Mammals are able to increase glucose production in the presence of glucagon. Current information suggest that fish respond similarly.

Goals

The main goal of this thesis is to improve current understanding of the hormonal regulation of glucose fluxes (R_a and R_d) in trout. The first part of my research (Chapter 2) focuses on the effects of insulin. The aim of this chapter is (1) to determine whether insulin acts the same way on glucose kinetics in fish as it does mammals; (2) to measure the levels of phosphorylated Akt and S6 in muscle and liver to assess whether the PI3K/Akt pathway is activated by insulin; (3) to measure circulating glucagon concentration to see if trout respond to insulin via a counter regulatory response by this hormone. The second part of my research (Chapter 3) focuses on the effects of glucagon. The goals of this chapter are (1) to determine whether glucagon affects R_a and R_d glucose the same way in fish as it does in mammals; (2) to measure the expression levels of important enzymes that regulate glycemia; (3) to measure the levels of phosphorylated PKA substrate and FOXO1 to establish whether the glucagon

signalling cascade is activated. Finally, Chapter 4 provides a general discussion of all the results to summarize what was learned about the hormonal regulation of glucose fluxes in rainbow trout.

Figure 1.1: Schematic representation of the two compartment model used for the Steele equations. The slower mixing pool is made of the intracellular volume of tissues and the rapid mixing pool is the plasma and extracellular fluid. Glucose production (R_a), Glucose disposal (R_d), and Infused labelled glucose (F).



CHAPTER 2
UNEXPECTED EFFECT OF INSULIN ON GLUCOSE DISPOSAL
EXPLAINS GLUCOSE INTOLERANCE IN TROUT

Based on a manuscript by the same title

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Introduction

Glucoregulation is generally considered essential to ensure adequate fuel supply to the brain and to working muscles (Shrayyef and Gerich, 2010; Wasserman et al., 2011). However, fish do not control glycemia as well as birds or mammals, and they do not rely on stable circulating glucose levels (Enes et al., 2009; Polakof et al., 2012). Carnivorous fish like salmonids are known to normalize glycemia very slowly in glucose tolerance tests (Legate et al., 2001), and they seem to show limited sensitivity to insulin (Marín-Juez et al., 2014). These observations are based on measurements of blood glucose concentration that depends on changing rates of glucose disposal (R_d) and hepatic glucose production (R_a). The hormonal regulation of *in vivo* glucose fluxes has been thoroughly characterized in mammals (Wasserman, 2009b), but remains unexplored in fish, except for the effects of epinephrine (Weber and Shanghavi, 2000). In humans, insulin can triple glucose disposal and almost completely suppress glucose production (Kelley et al., 1988; Lucidi et al., 2010). It also decreases glycemia in fish (Polakof et al., 2012), but this response could be mediated through a decrease in R_a , an increase in R_d , or both. Present evidence suggests that fish glucose production is probably inhibited by insulin because gluconeogenic enzymes are downregulated (Foster and Moon, 1990b; Polakof et al., 2010a; Polakof et al., 2010c). The effects of insulin on the R_d glucose of fish are more difficult to predict. It is possible that R_d is stimulated because insulin increases the expression of fish glucose transporters (GLUTs) in liver and muscle (Polakof et al., 2012; Polakof et al., 2010c). However, it is unclear how the glycolytic enzymes of these two key tissues respond because some studies show inhibition of expression (Polakof et al., 2010a; Polakof et al., 2010c), while

others report the opposite (Enes et al., 2009; Polakof et al., 2010b). Using continuous tracer infusion to quantify the respective impacts of the hormone on R_a and R_d glucose would clarify this issue.

The effects of insulin on glucose metabolism are mediated through several signalling cascades, but the PI3K/Akt pathway is the most important among them (Castillo et al., 2006). Insulin binding causes the phosphorylation of tyrosine residues in the β -subunit of the receptor, thereby activating a series of adaptor proteins (Caruso and Sheridan, 2011). In turn, these adaptor proteins cause the downstream activation of Akt and S6: two key elements of the PI3K/Akt signalling pathway. Phosphorylated forms of Akt and S6 are associated with the regulation of downstream metabolic processes that include glycogen synthesis, gluconeogenesis and cell growth (Caruso and Sheridan, 2011; Polakof et al., 2010a; Polakof et al., 2010c; Taniguchi et al., 2006). How intravascular insulin infusion affects Akt and S6 has not been measured.

The main goal of this study was to test the hypothesis that insulin reduces glycemia in fish by inhibiting glucose production and stimulating glucose disposal as it does in mammals. However, I anticipated that insulin would have a weaker effect on the glucose fluxes of fish than mammals because fish have a lower capacity for glucoregulation. Our secondary goals were: (i) to measure the levels of phosphorylated Akt and S6 in muscle and liver to determine whether the PI3K/Akt pathway is activated by insulin in these tissues, and (ii) to monitor circulating glucagon levels to see if insulin triggers a counterregulatory response.

Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) of both sexes with a Fulton's condition factor K of 1.15 ± 0.03 (N=22) [$K = (10^5 \times M_b) / L^3$]; where M_b = body mass in g and L = total body length in mm (Blackwell et al., 2000)] were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Two groups of fish were used: (i) for *in vivo* measurements of glucose kinetics by continuous tracer infusion, and (ii) for measurements of insulin signalling protein activation by Western blots (physical characteristics for each experimental group are in Table 2.1). The fish were held in a 1,200 liter flow-through tank supplied with dechloraminated Ottawa tap water at 13°C, on a 12 h:12 h light:dark photoperiod and were fed Profishnet floating fish pellets (Martin Mills, Elmira, ON, Canada) 5 days a week. They were acclimated to these conditions for a minimum of 2 weeks before experiments. All the procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care.

Catheterization and respirometry

Fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (60 mg l⁻¹ MS-222 buffered with 0.2 g l⁻¹ sodium bicarbonate) and doubly cannulated (for glucose kinetics experiments) or singly cannulated (for signalling protein experiments) with BTPE-50 catheters (Instech Laboratories, Plymouth Meeting, PA, USA) in the dorsal aorta (Haman and Weber, 1996). The catheters were kept patent by flushing with

Cortland saline containing 50 U ml⁻¹ heparin (Sigma-Aldrich, St Louis, MO, USA). Fish were left to recover overnight in a 90 liter swim-tunnel respirometer (Loligo Systems, Tjele, Denmark) where all measurements were carried out in resting animals at a water velocity of 0.5 body length per second (BL s⁻¹). This weak current reduces stress and enhances the flow of water over the gills, but does not require swimming to maintain body position while resting at the bottom of the chamber (Choi and Weber, 2015). The respirometer was supplied with the same quality water as the holding tank and kept at 13°C. Metabolic rate ($\dot{M}O_2$) was measured by intermittent flow respirometry using galvanic oxygen probes connected to a DAQ-PAC-G1 instrument controlled with AutoResp software (Loligo Systems). The probes were calibrated before each experiment using air saturated water (20.9% O₂).

Glucose kinetics experiments

Both catheters were made accessible through the respirometer lid by channeling them through a water-tight port. The rates of glucose production (R_a) and glucose disposal (R_d) were measured by continuous infusion of [6-³H]glucose (Perkin Elmer, Boston, MA, USA; 222 GBq mmol⁻¹). This tracer method has been validated to quantify glucose kinetics in fish (Haman et al., 1997a) and saline infusion has been used to validate no significant changes occur during the tracer method (Weber and Shanghavi, 2000). The infusate was freshly prepared immediately before each experiment by drying an aliquot of the solution obtained from the supplier under N₂ and resuspending in Cortland saline. A priming dose of tracer equivalent to 3 h of infusion was injected as a bolus at the start of each infusion to reach isotopic steady state in <45 min. The infusate

was then administered continuously at ~1 ml/h (determined individually for each fish to account for differences in body mass) using a calibrated syringe pump (Harvard Apparatus, South Natick, MA). Infusion rates for labeled glucose averaged 2180 ± 107 Bq kg⁻¹ min⁻¹ (N=10) and these trace amounts accounted for 0.00001% of the baseline rate of hepatic glucose production and does not change blood glucose concentration. Blood samples (~100 µl each) were drawn 50, 55 and 60 min after starting the tracer infusion to determine baseline glucose kinetics, and every 20 min thereafter during bovine insulin (Sigma Aldrich, Oakville, Ontario, Canada; I1882) administration (1.5 µg insulin kg⁻¹ min⁻¹ for 4 h). Bovine insulin has been used repeatedly to understand the actions of teleost insulin *in vivo* and *in vitro* (Ottolenghi et al., 1982; Polakof et al., 2010a; Polakof et al., 2010b; Polakof et al., 2010c; Van Raaij et al., 1995). The blood sampling schedule is indicated by arrows in Fig. 2.1. The amount of blood sampled from each fish accounted for <10% of total blood volume. Samples were collected in tubes containing heparin and aprotinin (500 KIU ml⁻¹ to stabilize glucagon). They were centrifuged to separate plasma (5 min; 12,000 RPM) that was stored at -20°C until analyses.

Signalling protein experiments

To avoid having to measure signalling proteins in radioactive tissues, these experiments were carried out on different fish than those used for glucose kinetics, but they received the same infusions: saline (control group) or insulin (treatment group; 1.5 µg kg⁻¹ min⁻¹) that were administered at 1 ml/h through the catheter for 4 h. The animals were then euthanized by a sharp blow on the head before collecting the liver and ~4 g

of white muscle anteriorly to the dorsal fin. The tissue samples were stored at -80 °C until analyses.

Sample analyses

Glucose kinetics experiments: Plasma glucose and glucagon concentrations were measured spectrophotometrically using a Spectra Max Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Glucagon was measured using a commercial ELISA kit (Crystal Chem, Downers Grove, IL). This kit uses a particular COOH-terminal anti-glucagon fragment that has been previously validated for fish glucagon (Navarro et al., 1995). Unfortunately, fish insulin cannot be measured accurately (Moon, 2001). A radioimmunoassay was developed decades ago (Plisetskaya, 1998), but it may also measure multiple pro-insulins and, therefore, greatly overestimates true insulin concentration. Glucose activity was quantified by drying plasma under N₂ to eliminate tritiated water and resuspending in distilled water. Radioactivity was then measured by scintillation counting (Perkin Elmer TriCarb 2910TR, Perkin-Elmer, Inc., Waltham, MA, USA) in Bio-Safe II scintillation fluid (RPI Corp., Mount Prospect, IL, USA).

Insulin signalling proteins experiments: Frozen livers and muscle (Control: N=6, Insulin: N=6; 200 mg) from the control and insulin infused rainbow trout were homogenized on ice with a sonicator (Fisher Scientific Sonic Dismembrator Model 100, San Diego, CA, USA) in 400 µl of buffer per 100 mg of tissue. During homogenization, samples were kept in a buffer containing 150 mmol l⁻¹ NaCl, 10 mmol l⁻¹ Tris, 1 mmol l⁻¹ EGTA, 1

mmol l⁻¹ EDTA (pH 7.4), 100 mmol l⁻¹ sodium fluoride, 4 mmol l⁻¹ sodium pyrophosphate, 2 mmol l⁻¹ sodium orthovanadate, 1% (v/v) Triton X-100, 0.5% (v/v) NP40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were centrifuged at 15,000 g for 5 min at 4°C, the resulting supernatants were recovered and stored at -80°C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (30 µg of total protein for liver and 50 µg of total protein for muscle) were subjected to SDS-PAGE (gel: 10% acrylamide/bis, proteins denatured at 95°C for 2 minutes) and western blotting (membrane: nitrocellulose) using the appropriate antibodies, tubulin, anti-p-Akt (S473), and anti-p-S6 (S235/236). The phosphorylated proteins, p-Akt and p-S6, were targeted because they represent the active form of the proteins and have shown activation in trout *in vitro* (hepatocytes and myocytes) and *in vivo* (Lansard et al., 2010; Mennigen et al., 2012; Polakof et al., 2011c; Seiliez et al., 2008). Membranes were incubated with Odyssey blocking buffer in phosphate buffered saline (PBS) to prevent nonspecific binding. Membranes were washed with PBS + 0.1% TWEEN20 then incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (v.3.0, LI-COR Biosciences). p-Akt and p-S6 protein intensity were normalized to beta tubulin intensity and expressed as relative fold change compared to control groups in liver and muscle tissues.

Calculations and statistics

Glucose fluxes were calculated in 2 different ways: (i) using either the steady-state, or (ii) the non-steady-state equations of Steele (Steele, 1959). Glucose turnover (R_t) was calculated using the steady-state equation. The nonsteady-state equations were used to calculate R_a and R_d glucose separately after changes in specific activity over time were curve-fitted by 2nd-degree polynomial regression for each animal (see Wolfe, 1992). Statistical comparisons were performed using one-way repeated-measures analysis of variance (RM-ANOVA) with the Dunnett's post hoc test to determine which means were significantly different from baseline (SigmaPlot v12, Systat Software, San Jose, CA, USA). When the assumptions of normality or equality of variances were not met, Friedman's non-parametric RM-ANOVA on ranks was used in cases where data transformations failed to normalize the data. Signalling protein levels were analyzed using Mann-Whitney rank sum test. Values are presented as means \pm s.e.m. and a level of significance of $P < 0.05$ was used in all tests.

Results

Metabolic rate

Metabolic rate (MO_2) was measured before and during insulin administration (Fig. 2.1). It remained stable at baseline values throughout the experiments ($P > 0.05$) and averaged $52.5 \pm 1.6 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$. Therefore, repeated blood sampling (indicated by arrows in Fig. 2.1) and the infusion of insulin had no stimulating effect on overall oxidative metabolism.

Glycemia and glucose kinetics

Changes in plasma glucose concentration and glucose specific activity over time are presented in Fig. 2.2. The administration of insulin caused a steady decrease in glucose concentration that became significant after 2 hours ($P < 0.05$; Fig. 2.2A). Glucose specific activity increased progressively from a mean baseline value of $240 \pm 20.8 \text{ Bq } \mu\text{mol}^{-1}$ to $452 \pm 58.8 \text{ Bq } \mu\text{mol}^{-1}$ after 4 h of insulin infusion ($P < 0.05$; Fig. 2.2B). The effects of insulin on glucose turnover rate (R_t), hepatic glucose production (R_a) and glucose disposal (R_d) are shown in Fig. 3. R_t glucose decreased progressively over the 4 h of insulin infusion ($P < 0.05$; Fig. 2.3A). The same decrease in glucose fluxes was observed when R_a and R_d glucose were calculated separately using nonsteady-state equations ($P < 0.05$; Fig. 2.3B). Differences between initial (baseline) and final values (after 4 h of insulin infusion) for glucose concentration and fluxes are summarized in Table 2.2. Final values for R_t , R_a and R_d glucose were significantly lower than the corresponding initial fluxes ($P < 0.001$). Figure 2.4 compares the effects of insulin on the glucose kinetics and glucoregulation capacity of fish and humans. Insulin inhibits R_d glucose in trout (-34%), but stimulates it in humans (+304%) (Fig. 2.4A). Insulin inhibits R_a glucose in trout (-38%) and suppresses it almost completely in humans (-99%) (Fig. 2.4B). Insulin weakly increases the capacity to lower glycemia [R_d glucose – R_a glucose] in trout to a maximal value of $0.5 \mu\text{mol kg}^{-1} \text{ min}^{-1}$. It stimulates this capacity much more strongly in humans where it reaches $43 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ (Fig. 2.4C). The $R_d - R_a$ ratio between humans and trout increases steadily during insulin administration (Fig. 2.4D). After a few hours of insulin infusion, the capacity to lower glycemia is 97 times higher in humans than in trout.

Glucagon

Changes in circulating glucagon concentration are presented in Fig. 2.5. Insulin caused a counter regulatory increase in glucagon that became significant after ~2 h ($P < 0.05$; Fig. 2.5). Initial and final glucagon concentrations are in Table 2.2.

Insulin signalling cascade

The effects of insulin on the active (phosphorylated) form of Akt and S6 in muscle and liver are shown in Fig. 2.6. In muscle, western blots reveal that both Akt (+4.6 fold; $P = 0.002$) and S6 (+7.2 fold; $P = 0.009$) are strongly activated by insulin. In the liver, fish receiving insulin were unable to elicit a significant activation of p-Akt and p-S6 compared to control animals ($P > 0.05$ Fig. 2.6).

Discussion

This study is the first to show that insulin has the opposite effect on the rate of glucose disposal of rainbow trout compared to mammals (Fig. 2.4A). Instead of stimulating R_d glucose to reduce glycemia rapidly, insulin inhibits glucose clearance from the circulation in trout. This explains why normalizing glycemia in glucose tolerance tests is about 10 times slower in trout than in mammals where insulin can triple R_d glucose (Legate et al., 2001). Results also show that insulin inhibits hepatic glucose production in trout, as it does in mammals. However, only partial reduction of R_a glucose was observed here in trout, whereas virtually complete insulin-mediated suppression of glucose production can be achieved by mammals (Shrayyef and Gerich,

2010). In trout, insulin reduces R_a glucose slightly more than R_d glucose (-43% vs -37%), and this small mismatch only allows a very slow reduction of glycemia. Hyperinsulinemia also causes a counterregulatory response in fish by raising glucagon levels and it activates the signalling proteins Akt and S6 in white muscle.

Inhibition of glucose disposal

Contrary to expectation, insulin inhibits glucose disposal in trout and, therefore, induces the opposite response classically seen in mammals (Figs. 2.3B and 2.4A). What mechanism could explain this striking difference? Glucose disposal can be modulated by altering intracellular glucose metabolism (glycolysis, glucose oxidation, glycogen synthesis) and/or transmembrane glucose transport that feeds these various pathways. Intracellular glucose phosphorylation by hexokinases and glucokinase plays an important role in the regulation of glycolytic flux (Panserat et al., 2014; Wasserman et al., 2011; Zhou et al., 2018). Therefore, the decrease in glucose disposal seen here in trout could be mediated through the reduced expression of muscle hexokinases and (liver) glucokinase, as previously reported in trout after insulin administration (Polakof et al., 2010a; Polakof et al., 2010c). The fact that insulin increases hexokinase and glucokinase expression, concentration, and activity in mammals (Panserat et al., 2014; Vogt et al., 1998) could explain why R_d glucose responds so differently between the two groups of animals. How the concentration and activity of these enzymes respond to insulin in trout has not been assessed directly, but present results suggest that they probably decrease. Slowing down phosphorylation causes an increase in intracellular free glucose that reduces the transmembrane concentration gradient driving inward

glucose transport via GLUTs. Surprisingly, reported responses for other aspects of glucose metabolism fail to explain why R_d glucose responds so differently between trout and mammals. They support the notion that trout R_d glucose should be stimulated by insulin because glycogen synthase activity (muscle) (Polakof et al., 2010c; Saltiel, 2015) and the expression of GLUTs (liver and muscle) (Polakof et al., 2010a; Polakof et al., 2010c; Saltiel, 2015) are stimulated by the hormone in both groups of animals. Unfortunately, the limited volume of blood that I could sample was insufficient to be able to assess potential responses from other hormones such as glucagon-like-peptide or growth hormone that might help to explain R_d glucose inhibition in trout. Overall, the information presently available suggests that the contrasting effects of insulin on the R_d glucose of trout and mammals depend on the opposite actions of the hormone on hexokinases.

Inhibition of glucose production

Insulin inhibits R_a glucose in trout and mammals, although less so in trout (Figs. 2.3B and 2.4B). Downregulating hepatic glucose production can be achieved by reducing glycogen breakdown, gluconeogenesis or both (Choi and Weber, 2015). The weaker R_a response of trout could be explained by the fact that insulin impairs glycogen breakdown in mammals, but may not do so in fish. The reciprocal activities of glycogen phosphorylase and glycogen synthase determine the rate of net glycogen synthesis or breakdown (Pereira et al., 1995). In mammals, insulin strongly inhibits glycogen phosphorylase and stimulates glycogen synthase (Dimitriadis et al., 2011; Shrayyef and Gerich, 2010), but these enzymes seem to be unresponsive in trout (Polakof et al.,

2010c). Therefore, trout probably maintain baseline glycogen breakdown when insulin is elevated, making the full suppression of R_a glucose impossible.

The weaker inhibiting effect of insulin on the R_a glucose of trout (compared to mammals) could also be linked to differences in the regulation of gluconeogenesis. Insulin clearly downregulates key gluconeogenic enzymes like phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase in mammals (Saltiel, 2015), and there is evidence that the same enzymes could be inhibited in trout (Polakof et al., 2010c). However, many gene duplication events have happened in the evolutionary history of trout, resulting in multiple forms of these enzymes. A recent study suggests that different gluconeogenic isoforms could be regulated differently (Marandel et al., 2015). If the results of Polakof et al (Polakof et al., 2010c) only apply to a single isoform, they may not express the net effect of insulin on gluconeogenic flux. Overall, therefore, the weak inhibition of glucose production shown by trout can be attributed to the lack of response by glycogen phosphorylase and glycogen synthase and, possibly, to the atypical regulation of gluconeogenic isoform expressions.

Capacity to clear glucose

Insulin decreases glycemia in both trout and mammals, but at vastly different rates (Lucidi et al., 2010; Polakof et al., 2012). The capacity of insulin to lower blood glucose can be expressed as the difference between disposal and production (R_d glucose - R_a glucose), and it is shown in Fig. 2.4 that compares humans with trout. Humans are rapidly able to achieve a $R_d - R_a$ difference of $43 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (Lucidi et

al., 2010), whereas the maximal value measured here in trout was only $0.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (Fig. 2.4C). After 90 min of insulin administration, the capacity to clear glucose was 97 times higher in humans than in trout (Fig. 2.4D), and this value is probably an underestimation of the true ratio because less insulin was given in the human experiments.

Such differences in the capacity to clear glucose may exist because carnivorous salmonids naturally consume a low carbohydrate diet and only experience hyperglycemia very rarely. Also, persistent hyperglycemia may not be as harmful to trout as mammals. In diabetic humans, it can cause protein glycosylation that leads to retinopathy, neuropathy, renal failure, and atrial fibrillation (Alberti and Zimmet, 1998; Yang et al., 2015), but it is unclear whether similar outcomes occur in trout. Chronic hyperglycemia can cause retinopathy in zebrafish (*Danio rerio*) (Gleeson et al., 2007), or hemoglobin glycosylation and insulin resistance in Indian perch (*Anabas testudineus*) (Barma et al., 2006). However, cave-dwelling fish populations of *Astyanax mexicanus* are hyperglycemic throughout their life and do not show any health complications (Riddle et al., 2018). Overall, the capacity to clear glucose rapidly is most likely not needed in rainbow trout because they normally eat little glucose and/or easily tolerate hyperglycemia.

Activation of insulin signalling pathway

Insulin was able to activate Akt and S6 in muscle (Fig. 2.6), but this stimulation of the PI3K/Akt-signalling pathway did not increase R_d glucose as it does in mammals.

Instead, trout R_d glucose decreased, and it is unclear why this should be the case. Most of the detailed information on this signalling pathway comes from non-piscine models and some, but not all of its components are known in fish (Caruso and Sheridan, 2011). The uncharacterized parts of the insulin signalling cascade of trout could therefore be quite different from mammals. The target genes or downstream enzymes regulated by the cascade could also have evolved differently. The opposite effects of insulin on gluco/hexokinases of trout (inhibition) and mammals (activation)(Polakof et al., 2010c) are somewhat puzzling. This is because these enzymes are modulated by sterol regulatory element-binding protein-1 (SREBP-1)(Ruiz et al., 2014) and insulin stimulates SREBP-1 in both trout and mammals (Lansard et al., 2010; Ruiz et al., 2014). SREBP-1 activation may have opposite effects on the expression of these kinases in trout vs mammals. Unknown components of the insulin signalling cascade or non-mammalian regulation of gluco/hexokinases by SREBP-1 could therefore explain why trout activate the muscle PI3K/Akt cascade while decreasing R_d glucose.

Insulin did not affect the liver PI3K/Akt cascade in our experiments (Fig. 2.6), but several other studies have reported activation (Plagnes-Juan et al., 2008; Polakof et al., 2010b; Polakof et al., 2010c). It is unclear why the liver failed to respond, but these discrepancies might be explained by varying modes of hormone administration: intraperitoneal bolus (IP) injection (Plagnes-Juan et al., 2008), 11 day-osmotic pump IP infusion (Polakof et al., 2010b), intravascular (IV) bolus injection (Polakof et al., 2010c), 4 h-IV infusion (this study). Therefore, differences in insulin concentrations around the

liver and in the time course of their changes may have affected whether the PI3K/Akt cascade responds or not.

Stimulation of glucagon release

Insulin injection resulted in a large increase in circulating glucagon concentration (Fig. 2.5), and this response could be mediated by hypoglycemia. Glucosensing neurons detect declining glucose levels (Polakof et al., 2011b) and could stimulate glucagon release to counteract the effects of insulin. Additionally, Glucagon increases the expression and release of somatostatins 14 and 25 (Ehrman et al., 2005), hormones that indirectly promote hyperglycemia by inhibiting insulin (Sheridan and Kittilson, 2004). Therefore, counterregulation of hyperinsulinemia probably also includes somatostatins in rainbow trout.

Conclusions and significance

This study is the first to characterize the integrated *in vivo* effects of insulin on fish glucose metabolism. It shows that rainbow trout have a unique response to the hormone: ***the inhibition of glucose disposal*** (Fig. 2.3). This unexpected effect of insulin has not been documented in other animals, especially mammals that stimulate R_d glucose by several fold instead (Fig. 2.4). These interspecific differences may be explained by the contrasting effects of insulin on the gluco / hexokinases of trout (inhibition) vs mammals (activation). Results also show that insulin reduces hepatic glucose production in trout, whereas mammals can achieve complete suppression. This partial reduction of R_a glucose may be because insulin does not affect glycogenolysis in

trout and only inhibits gluconeogenesis, whereas mammals shut down both pathways. The integrated actions of insulin that lead to reducing glucose fluxes in trout (R_a slightly more than R_d) only provide them with a very limited capacity to decrease glycemia because $R_d - R_a$ remains extremely small. I conclude that the glucose intolerance classically exhibited by rainbow trout can be partially explained by the inhibiting effect of insulin on glucose disposal.

Table 2.1: Mean physical characteristics and hematocrit of the 2 groups of catheterized rainbow trout used (i) for *in vivo* measurements of glucose kinetics or (ii) for tissue measurements of insulin signalling proteins. Values are means \pm s.e.m (sample sizes are in parentheses).

	Glucose kinetics experiments (10)	Signalling proteins experiments (12)
Body mass (g)	452 ± 23	322 ± 29
Body length (cm)	33 ± 0.4	30.8 ± 0.7
Hematocrit (%)	20.1 ± 0.6	19.6 ± 0.4

Table 2.2: Initial (baseline) and final values after 4 h of insulin administration for various parameters of glucose metabolism and for circulating glucagon in rainbow trout. Values are means \pm s.e.m (N=10). Glucose turnover rate (R_t) was obtained with the steady state equation, whereas the rates of appearance (R_a) and disposal (R_d) were obtained with the non-steady state equations of Steele (Steele, 1959). The effects of insulin are indicated as * $P < 0.01$ or ** $P < 0.001$ (paired t-test).

	Baseline	Final (after 4 h of insulin infusion)
Glucose ($\mu\text{mol ml}^{-1}$)	5.5 ± 0.5	3.9 ± 0.3 *
R _t Glucose ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	9.6 ± 0.8	5.5 ± 0.7 **
R _a Glucose ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	8.5 ± 0.7	4.8 ± 0.6 **
R _d Glucose ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	8.6 ± 0.6	5.4 ± 0.5 **
Glucagon (pg ml^{-1})	26.5 ± 12.7	216.4 ± 97.6 *

Fig. 2.1. Experimental design and metabolic rate (MO_2) of resting rainbow trout during the measurement of glucose kinetics. Insulin administration started at time 0 and lasted 4 h. Glucose kinetics were quantified before and during insulin administration by continuous infusion of $[6-^3H]$ glucose that started at time -1h. Arrows indicate when blood samples were collected. MO_2 values are means \pm s.e.m. (N=10). Blood sampling and insulin had no effect on MO_2 ($P=0.3$; one-way RM ANOVA).

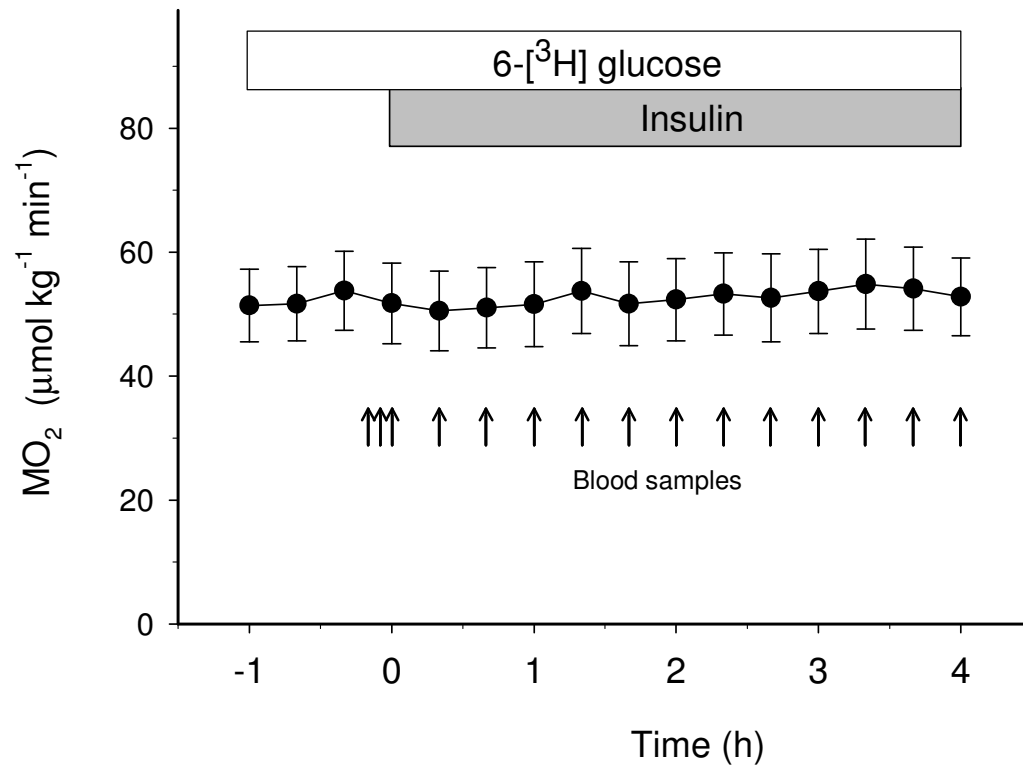


Fig. 2.2. Effects of insulin on plasma glucose concentration (panel A) and glucose specific activity (panel B). Values are means \pm s.e.m. (N=10). Means significantly different from baseline are indicated by * ($P<0.05$; one-way RM ANOVA).

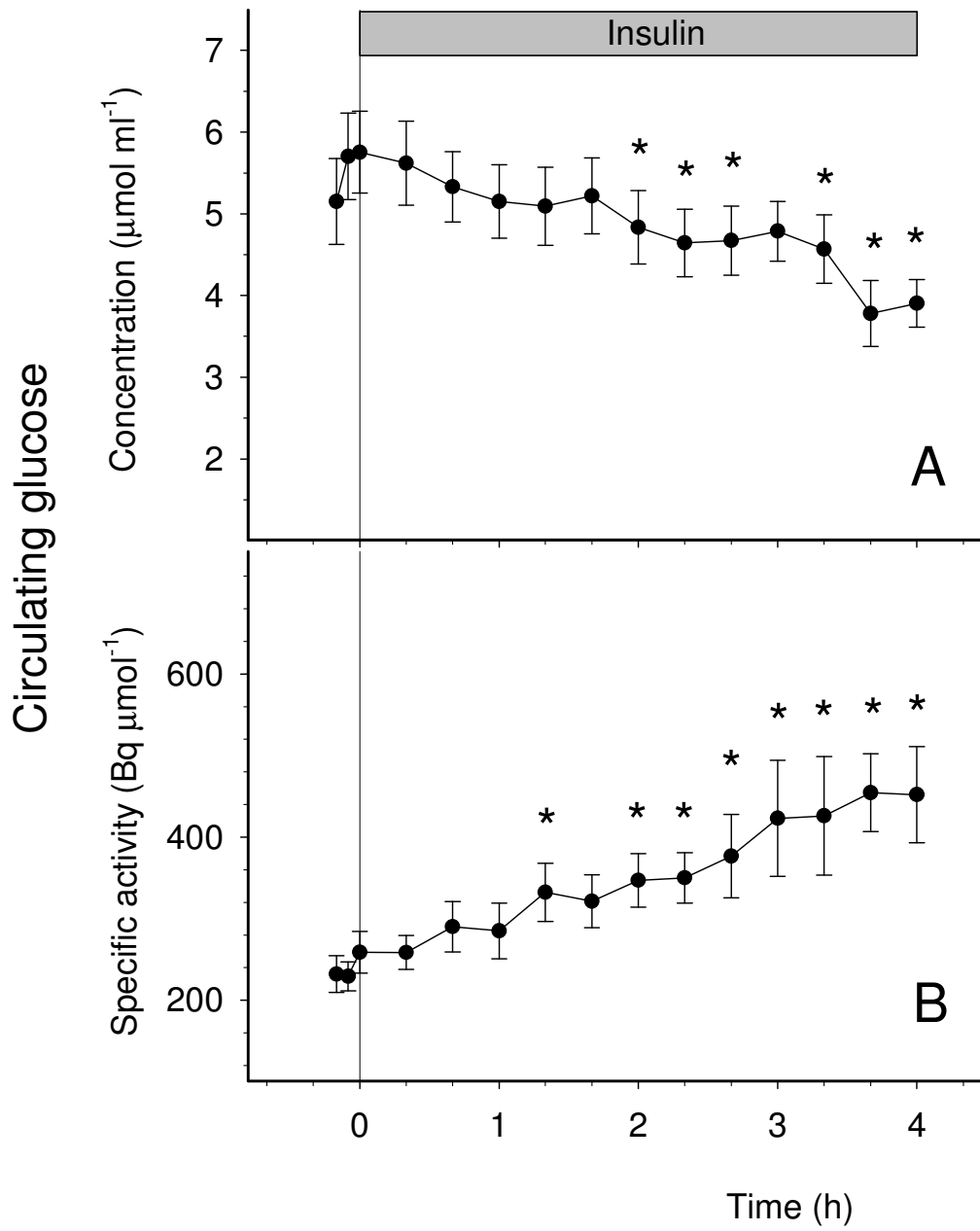


Fig. 2.3. Effects of insulin on the glucose fluxes of rainbow trout. Fluxes were either calculated with the steady-state equation [turnover rate (R_t), panel A] or the nonsteady-state equations of Steele [glucose disposal (R_d) and glucose production (R_a), panel B] (Steele, 1959). Values are means \pm s.e.m. (N=10). Means significantly different from baseline are indicated by * ($P<0.05$; one-way RM ANOVA).

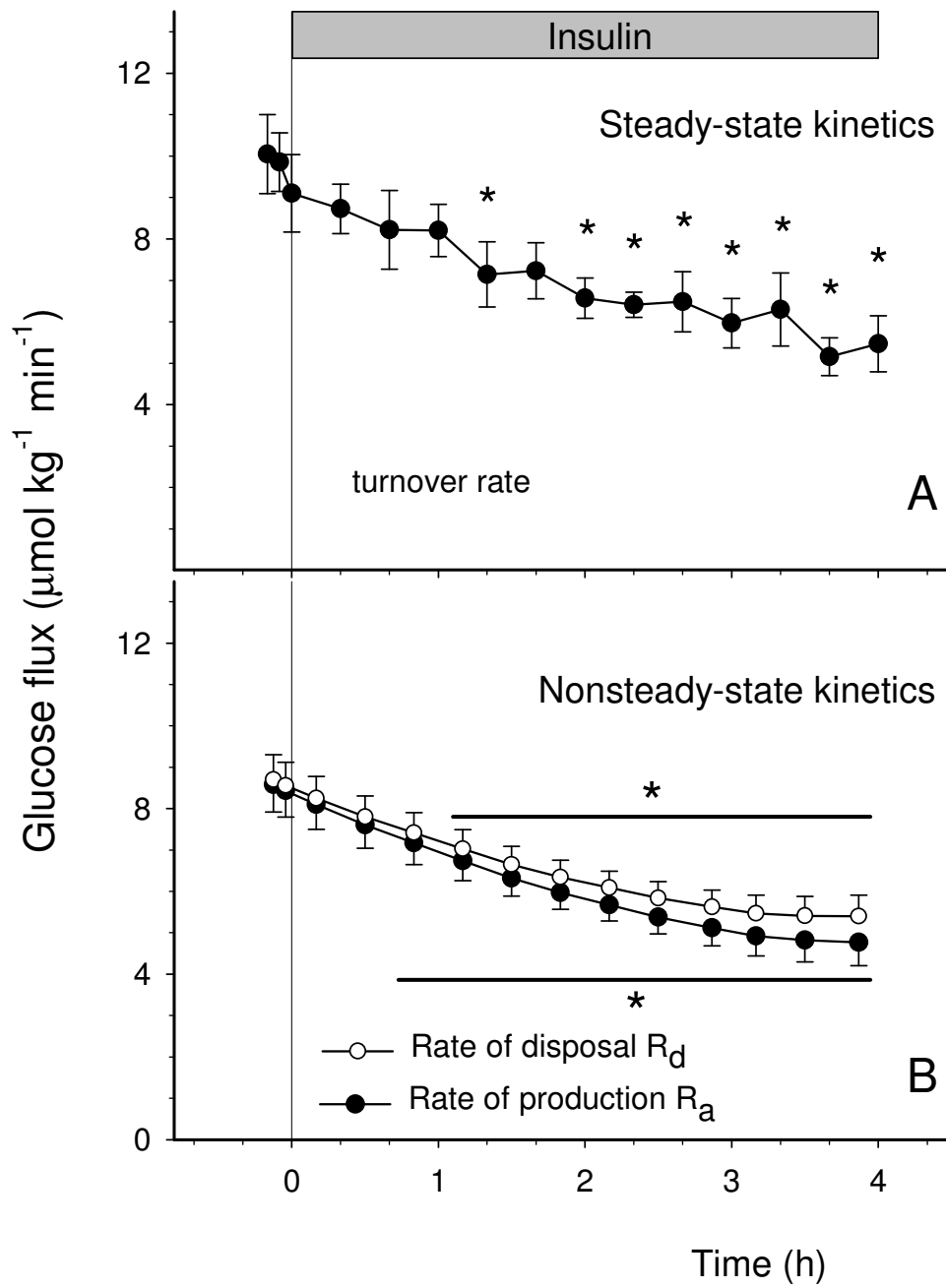


Fig. 2.4. Comparative effects of insulin on the glucose fluxes of rainbow trout and humans. Fish values are compared with human results adapted from Lucidi et al (Lucidi et al., 2010): a similar study where insulin was administered continuously for 3 h. **A:** Relative changes in R_d glucose; **B:** Relative changes in R_a glucose; **C:** Effects of insulin on the capacity to lower glycemia expressed as R_d glucose – R_a glucose; **D:** Changes in the capacity to lower glycemia ($R_d - R_a$) ratio between humans and trout during insulin administration.

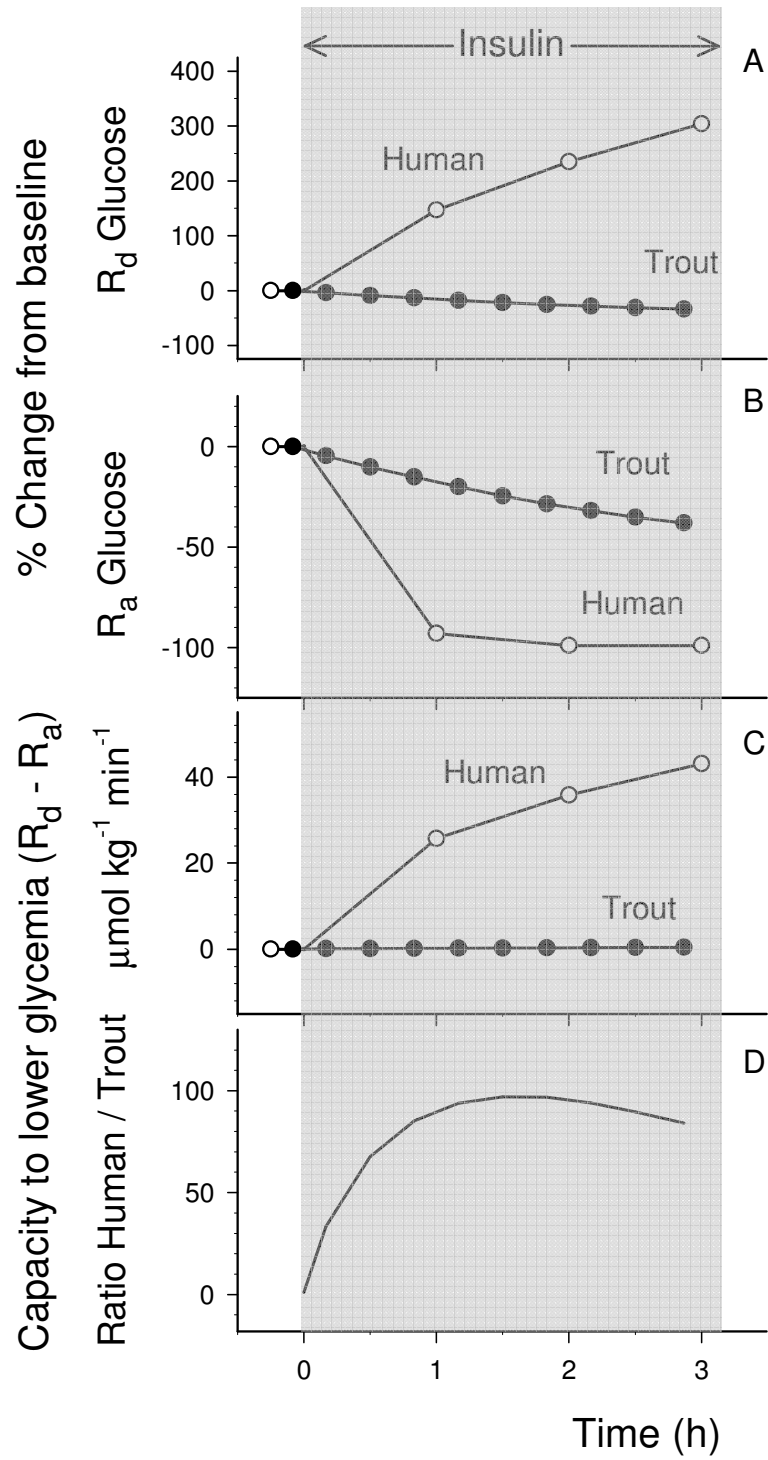


Fig. 2.5. Effects of insulin on circulating glucagon levels in rainbow trout. Values are means \pm s.e.m. (N=10). Means significantly different from baseline are indicated by * ($P<0.05$; one-way RM ANOVA).

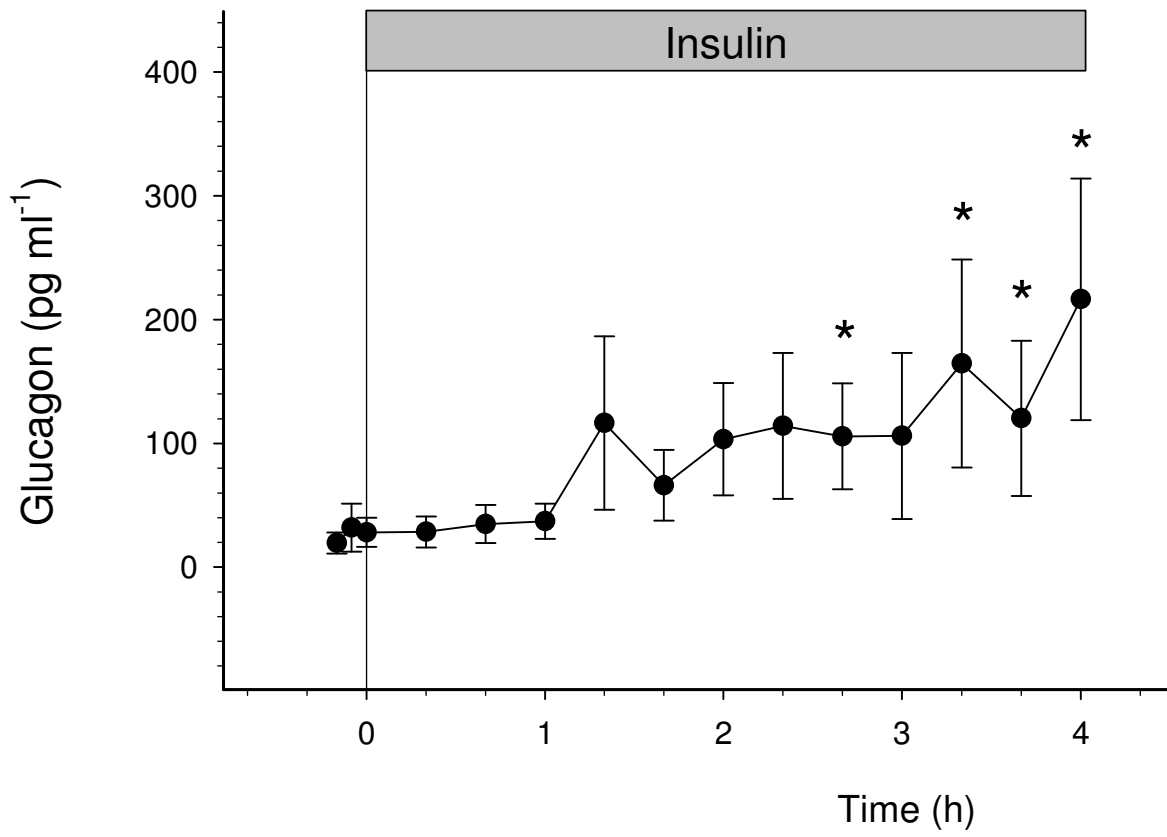
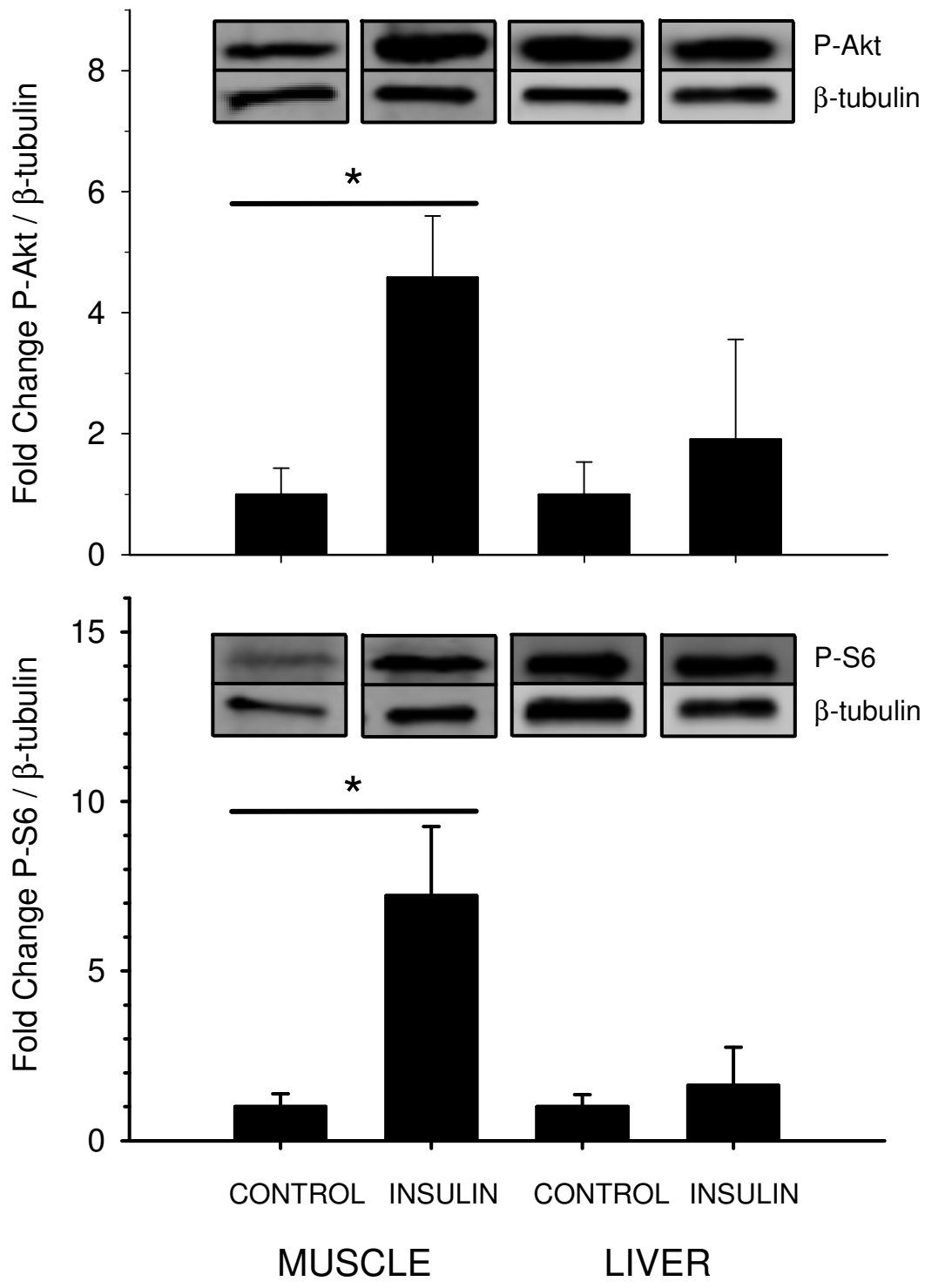


Fig. 2.6. Relative effects of insulin on the levels of key signalling proteins. For each mean, western blots are given for the phosphorylated protein (top) and β -tubulin (bottom). Values are means \pm s.e.m. (N=6 for each group). Significant insulin activation is indicated by * ($P<0.01$; Mann-Whitney rank sum test).



CHAPTER 3
REGULATION OF RAINBOW TROUT GLUCOSE FLUXES BY GLUCAGON

Based on a manuscript by a similar title

Written by

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Not submitted

Author contributions: J.F. and J.-M.W. conception and design of research; J.F. performed experiments; J.F. and D.K. analyzed data; J.F. and J.-M.W. interpreted results of experiments; J.F., D.K., J.M. and J.-M.W prepared figures; J.F. drafted manuscript; J.F. and J.-M.W. edited and revised manuscript; J.F. and J.-M.W. approved final version of manuscript.

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Introduction

The regulation of circulating glucose levels allows adequate fuel supply to the brain and to working muscles (Shrayyef and Gerich, 2010; Wasserman et al., 2011). Mammals and birds tightly control glycemia, but fish do not (Enes et al., 2009; Polakof et al., 2012). Blood glucose concentration depends on changing rates of hepatic glucose production (R_a glucose) and glucose disposal (R_d glucose). The hormonal regulation of *in vivo* glucose fluxes has been thoroughly characterized in mammals (Wasserman, 2009a), but remains relatively unexplored in fish. The catabolic hormone glucagon is one of the two key endocrine signals regulating glucose fluxes in mammals and possibly also in fish (Polakof et al., 2012; Shrayyef and Gerich, 2010). Multiple metabolic effects of glucagon have been investigated in mammals, but little information is currently available for fish. In humans, glucagon stimulates glucose production and weakly inhibits (Lins et al., 1983) or has no effect (Hinshaw et al., 2015) on glucose disposal. Glucagon increases glycemia in fish, as in mammals (Polakof et al., 2012), but this response could be mediated through an increase in R_a glucose, a decrease in R_d glucose, or both. Current information suggests that fish R_a is probably also stimulated by glucagon because gluconeogenic and glycogenolytic enzymes are upregulated by this hormone in hepatocytes (Brighenti et al., 1991; Foster and Moon, 1990a; Puviani et al., 1990; Sugita et al., 2001). Several studies support the notion that fish R_d glucose should be inhibited because glucagon decreases liver activities of glycogen synthase (Murat and Plisetskaya, 1977), phosphofructokinase (Foster et al., 1989) and pyruvate kinase (Petersen et al., 1987). Direct measurement of the effects of glucagon on *in vivo*

glucose kinetics is needed to establish how fish R_a and R_d glucose respond to the hormone.

The effects of glucagon on glucose metabolism are mediated through two pathways: cAMP and calcium signalling. Glucagon binds to the G-protein coupled receptor that raises GTP levels, thereby increasing cAMP and IP_3 (Moon et al., 1997). In turn, the cAMP and calcium signalling pathways are mobilized resulting in the activation of protein kinase A (PKA) substrates (Plisetskaya and Mommsen, 1996) and possibly FoxO1 like in mammals (Eijkelenboom and Burgering, 2013). Phosphorylated forms of PKA substrates and FoxO1 are associated with the regulation of downstream metabolic processes that include glycogen breakdown and gluconeogenesis (Habegger et al., 2010; Moon, 1998). Whether glucagon activates PKA substrates and FoxO1 in fish has not been determined and it would be especially useful to know this for tissues like liver and muscle that play a key role in glucose metabolism.

The main goal of this study was to test the hypothesis that glucagon increases glycemia in fish by stimulating hepatic glucose production and inhibiting glucose disposal. I anticipated that glucagon would have a weaker effect on the hepatic glucose production of fish than mammals because fish are generally thought to have a lower capacity for glucoregulation. Our secondary goals were: (i) to measure the levels of phosphorylated PKA substrates and FoxO1 in muscle and liver to determine whether the glucagon signalling cascade is activated in these tissues, and (ii) to measure transcript levels of key proteins involved in gluconeogenesis, glycogen breakdown,

glycolysis, and transmembrane glucose transport. Different protein isoforms will be investigated in gluconeogenesis and glycogen breakdown because salmonids have multiple isoforms of phosphoenolpyruvate carboxykinase (Pepck) and glycogen phosphorylase (Gp) (Marandel et al., 2015).

Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) of both sexes with a Fulton's condition factor K of 1.09 ± 0.02 (N=22) [$K = (10^5 \times M_b) / L^3$]; where M_b = body mass in g and L = total body length in mm (Blackwell et al., 2000)] were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Two groups of fish were used: (i) for *in vivo* measurements of glucose kinetics by continuous tracer infusion, and (ii) for measurements of glucagon signalling protein activation by Western blots and gene expression of enzymes by qPCR (physical characteristics for each experimental group are in Table 3.1). The fish were held in a 1,200 liter flow-through tank supplied with dechloraminated Ottawa tap water at 13°C, on a 12 h:12 h light:dark photoperiod and were fed Profishnet floating fish pellets (Martin Mills, Elmira, ON, Canada) 5 days a week. They were acclimated to these conditions for a minimum of 2 weeks before experiments. All the procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care.

Catheterization and respirometry

Refer to Chapter 2

Glucose kinetics experiments

Both catheters were made accessible through the respirometer lid by channeling them through a water-tight port. The rates of glucose production (R_a) and glucose disposal (R_d) were measured by continuous infusion of [$6\text{-}^3\text{H}$]glucose (Perkin Elmer, Boston, MA, USA; 222 GBq mmol^{-1}). This tracer method has been validated to quantify glucose kinetics in fish (Haman et al., 1997a). The infusate was freshly prepared immediately before each experiment by drying an aliquot of the solution obtained from the supplier under N_2 and resuspending in Cortland saline. A priming dose of tracer equivalent to 3 h of infusion was injected as a bolus at the start of each infusion to reach isotopic steady state in <45 min. The infusate was then administered continuously at ~ 1 ml/h (determined individually for each fish to account for differences in body mass) using a calibrated syringe pump (Harvard Apparatus, South Natick, MA). Infusion rates for labeled glucose averaged $2007 \pm 181\text{ Bq kg}^{-1}\text{ min}^{-1}$ ($N=8$) and these trace amounts accounted for 0.00001% of the baseline rate of hepatic glucose production. Blood samples ($\sim 100\text{ }\mu\text{l}$ each) were drawn 50, 55 and 60 min after starting the tracer infusion to determine baseline glucose kinetics, and every 20 min thereafter during bovine glucagon (Prospec, Rehovot, Israel; HOR-286) administration ($8.3\text{ }\mu\text{g glucagon kg}^{-1}\text{ min}^{-1}$ for 4 h). Bovine glucagon has been used repeatedly to understand the actions of teleost glucagon (Foster and Moon, 1990a; Foster et al., 1989; Plisetskaya and Mommsen, 1996). The amount of blood sampled from each fish accounted for $<10\%$ of total blood volume. Samples were collected in tubes containing heparin and aprotinin

(500 KIU ml⁻¹ to stabilize glucagon). They were centrifuged to separate plasma (5 min; 12,000 RPM) that was stored at -20°C until analyses.

Signalling protein experiments

To avoid having to measure signalling proteins in radioactive tissues, these experiments were carried out on different fish than those used for glucose kinetics, but they received the same infusions: saline (control group) or glucagon (treatment group; 8.3 µg kg⁻¹ min⁻¹) that were administered at 1 ml/h through the catheter for 4 h. The animals were then euthanized by a sharp blow on the head before collecting the liver and ~4 g of white muscle anteriorly to the dorsal fin. The tissue samples were stored at -80°C until analyses.

Sample analyses

Glucose kinetics: Refer to Chapter 2

Glucagon signalling proteins experiments: Frozen livers and muscle (Control: N=7 Glucagon: N=7; 200 mg) from the control and glucagon infused rainbow trout were homogenized on ice with a sonicator (Fisher Scientific Sonic Dismembrator Model 100, San Diego, CA, USA) in 400 µl of buffer per 100 mg of tissue. During homogenization, samples were kept in a buffer containing 150 mmol l⁻¹ NaCl, 10 mmol l⁻¹ Tris, 1 mmol l⁻¹ EGTA, 1 mmol l⁻¹ EDTA (pH 7.4), 100 mmol l⁻¹ sodium fluoride, 4 mmol l⁻¹ sodium pyrophosphate, 2 mmol l⁻¹ sodium orthovanadate, 1% (v/v) Triton X-100, 0.5% (v/v) NP40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were centrifuged at 15,000 g for 5 min at 4°C, the resulting supernatants

were recovered and stored at -80°C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (200 µg of total protein for liver and 50 µg of total protein for muscle) were subjected to SDS-PAGE (gel: 10% acrylamide/bis, denatured at 95°C for 2 minutes) and western blotting (membrane: nitrocellulose) using the appropriate antibodies, anti-p-PKA substrates (RRXS*/T*), anti-p-FOXO1 and were normalized using REVERT Total Protein Stain. Odyssey blocking buffer phosphate buffered saline (PBS) was used to prevent nonspecific binding. Membranes were washed with PBS + 0.1% TWEEN20 then incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (v.3.0, LI-COR Biosciences).

Analysis of mRNA transcript abundance: Total RNA was extracted from 20 to 100 mg of liver or muscle using TRIzol (Invitrogen, Burlington, ON, Canada) following the manufacturer's protocol. Tissues (Control: N=7 Glucagon: N=7) were homogenized using a sonicator until tissue fragments were no longer visible. Extracted RNA was quantified using a NanoDrop® 2000c UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Ottawa, ON, Canada). Next cDNA was generated using a QuantiTech Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's protocol. Two step semi-quantitative real-time RT-PCR assays were performed on a Bio-Rad CFX96 instrument (Bio-Rad, Mississauga, ON, Canada) to quantify fold changes in relative liver and muscle mRNA abundances of key transcripts involved in gluconeogenesis (*pck1*, *pck2a*, *pck2b*, *fbpase*), glycogenolysis (*gpb*, *gpmuscle*,

g6pase), glycolysis (*pfk*) and glucose transport (*glut1bb*, *glut2*, *glut4*). A standard curve consisting of serial dilutions of pooled cDNA, a negative no-RT control consisting of cDNA generated in a reaction that did not include reverse transcriptase, a negative no-template control generated in a reaction that substituted water for RNA and individual diluted samples were run in duplicate. For each individual reaction, the total volume was 20 μ l, that consisted of 4 μ l diluted cDNA template, 0.5 μ l 10 μ M specific forward and 0.5 μ l 10 μ M specific reverse primer (Table 3.3), 10 μ l SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 5 μ l nuclease free H₂O. For each assay, cycling parameters were a 2 min activation step at 98°C followed by 40 cycles consisting of a 20 sec denaturation step at 95°C and a combined 30 sec annealing and extension step at primer specific temperatures (Table 3.3). Following each run, melting curves were produced (65°C – 95°C at 0.5°C every 5 seconds) by gradually increasing temperature and the final curves were monitored for single peaks to confirm the specificity of the reaction and the absence of primer dimers. In cases where primers were newly designed, pooled samples were sent for sequencing (Ottawa Hospital Research Institute, Ottawa, ON, Canada), followed by BLAST search (NCBI), to confirm amplicon specificity. The acceptable range for amplification efficiency calculated from serially diluted standard curves was 90-110% with R² values >0.97. Assays were subsequently normalized using the NORMA-Gene approach as described by Heckmann et al. (Heckmann et al., 2011). Finally, mRNA fold-changes were calculated relative to the control group.

Calculations and statistics

Glucose fluxes were calculated in 2 different ways: using either the steady-state, or the non-steady-state equations of Steele (Steele, 1959). Glucose turnover (R_t) was calculated using the steady-state equation. The nonsteady-state equations were used to calculate R_a and R_d glucose separately after changes in specific activity over time were curve-fitted by 2nd-degree polynomial regression for each animal (see Wolfe, 1992). Statistical comparisons were performed using one-way repeated-measures analysis of variance (RM-ANOVA) with the Dunnett's post hoc test to determine which means were significantly different from baseline (SigmaPlot v12, Systat Software, San Jose, CA, USA). When the assumptions of normality or equality of variances were not met, Friedman's non-parametric RM-ANOVA on ranks was used because data transformations were unsuccessful. Signalling protein data and gene expression data were analyzed using an unpaired t-test and when normality and equal variances were not met, Mann-Whitney rank sum test was used. Values are presented as means \pm s.e.m. and a level of significance of $P < 0.05$ was used in all tests.

Results

Glycemia and glucose kinetics

Fig. 3.1 shows the changes in plasma glucose concentration and glucose specific activity during 4 h of glucagon administration. Glucose concentration increased progressively for the first 3 h before reaching a plateau during the last hour of the experiment ($P < 0.05$; Fig. 3.1A). Glucose specific activity showed no significant change from baseline ($P > 0.05$; Fig. 3.1B). The effects of glucagon on glucose turnover rate (R_t), hepatic glucose production (R_a), glucose disposal (R_d) and on the capacity to increase

glycemia [R_a glucose – R_d glucose] are presented in Fig. 3.2. R_t glucose showed no significant response to the hormone ($P>0.05$; Fig. 3.2A). Both R_a and R_d glucose remained constant for the first 2 h of glucagon infusion before decreasing progressively to reach a significantly lower value than baseline after 4 h ($P<0.05$; Fig. 3.2B). The capacity to increase glycemia [R_a glucose – R_d glucose] was stimulated to a maximum of $1.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$ during the first few min of glucagon infusion before decreasing steadily to zero over the course of the experiment ($P<0.05$; Fig. 3.2C). Table 3.2 summarizes initial (baseline) values and final values (after 4 h of glucagon infusion) for glucose concentration and glucose fluxes.

Gene expression

The effects of glucagon on muscle and liver mRNA abundance of key proteins in glucose metabolism are presented in Figs. 3.3 and 3.4. In muscle, glucagon caused an increase in glycogen phosphorylase isoform *glycogen phosphorylase b (gpb)* ($P<0.01$; Fig. 3.3A) and a decrease in glycogen phosphorylase isoform *glycogen phosphorylase muscle (gpmuscle)* ($P<0.05$; Fig. 3.3C). No significant changes were observed in *phosphofructokinase-1 (pfk1)*, and *glucose transporters 1 and 4 (glut1 and glut4)* ($P>0.05$; Fig. 3.3B, 3.3D, and 3.3E). In liver, *phosphoenolpyruvate carboxykinase 1 (pck1)* increased over 40-fold ($P<0.01$; Fig. 3.4A) and *pck2a* decreased ($P<0.05$; Fig. 3.4E). Liver *g6pase*, *fbpase*, *glut2*, and *pck2b* were unaffected by glucagon ($P>0.05$; Fig. 3.4B, 3.4C, 3.4D and 3.4F).

Circulating glucagon

Changes in plasma glucagon concentration are presented in Fig. 3.5. The concentration of the hormone increased progressively throughout the experiment and reached values significantly higher than baseline after 2 hours ($P<0.05$; Fig. 3.5).

Glucagon signalling cascade

The effects of glucagon on the active (phosphorylated) form of PKA substrates and FoxO1 in muscle and liver are shown in Fig. 3.6. Glucagon did not significantly activate PKA substrates in muscle ($P=0.933$) or liver ($P=0.131$). Similarly, it did not activate FoxO1 of either tissue (muscle: $P=0.212$; liver: $P=0.294$).

Discussion

This study provides the first *in vivo* measurements of glucose fluxes in rainbow trout during glucagon administration. It shows that this hormone elicits moderate hyperglycemia by causing a temporary mismatch between glucose production (non-significant increase) and utilization (non-significant decrease) that progressively disappears over 4 h (Figs. 3.1 and 3.2). Surprisingly, both R_a and R_d end up decreasing during the last 2 h of glucagon administration, and eventually reach lower values than baseline. Results also reveal that glucagon regulates the 3 known isoforms of fish Pepck differently at the transcription level (Fig. 3.4). Furthermore, the expression of glycogen phosphorylase transcripts is modulated differently between tissues, and even within muscle: upregulation of *gpb* and downregulation of *gpmuscle* by glucagon (Fig. 3.5).

Initial effects of glucagon on glucose fluxes

Increasing circulating glucagon causes an initial divergence between R_a and R_d that raises blood glucose concentration by 42%. These immediate responses (stimulation of R_a and inhibition of R_d) fail to reach statistical significance (Fig. 3.2B), but clearly occur because they are necessary to explain the observed hyperglycemia (Fig. 3.1A), and the difference between R_a and R_d jumps to $1.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$ immediately upon glucagon administration (Fig. 3.2C). Increasing hepatic glucose production can be achieved by stimulating gluconeogenesis, glycogen breakdown or both (Choi and Weber, 2015). Previous fish studies show that glucagon stimulates gluconeogenesis by increasing Pepck expression (Foster and Moon, 1990a) and by increasing FBPase and G6Pase activity in isolated hepatocytes (Sugita et al., 2001). Similar responses have been documented for mammals that increase the expression and activity of these gluconeogenic enzymes (Band and Jones, 1980; Beale et al., 1984; Christ et al., 1988; Pilkis et al., 1982; Striffler et al., 1984). In addition, glucagon stimulates glycogen breakdown by increasing glycogen phosphorylase activity by ~50% (Puviani et al., 1990) in trout, and ~160% in mammals (Malbon et al., 1978). Glucagon increases R_a glucose by stimulating gluconeogenesis and glycogen breakdown in trout, but the hormone has a weaker effect than in mammals. Mechanisms to decrease glucose disposal are less clear, but inhibition of glycogen synthesis and hepatic glycolysis are most likely involved. Experiments on fish liver have shown that glucagon reduces the activities of glycogen synthase (Murat and Plisetskaya, 1977), pyruvate kinase (Pk)(Petersen et al., 1987) and phosphofructokinase (Pfk)(Foster et al., 1989), similarly to what is known to occur in mammals (Jiang and Zhang, 2003). Overall, therefore, the

glucagon-driven hyperglycemia observed here is probably due to multiple liver responses that include the stimulation of gluconeogenesis and glycogen phosphorylase, and the inhibition of glycogen synthase and glycolysis.

Longer term effects of glucagon

After two hours of glucagon infusion, blood glucose concentration stabilizes at about $12 \mu\text{mol ml}^{-1}$ (Fig. 3.1A), glucose fluxes decrease progressively below baseline (Fig. 3.2B), and the capacity to raise glycemia [$R_a - R_d$] approaches zero (Fig. 3.2C). These results suggest that glucagon triggers a counterregulatory response by insulin in an attempt to restore normoglycemia. When fish experience hyperglycemia, glucosensing neurons and beta cells of the Brockmann bodies stimulate insulin secretion (Blasco et al., 2001; de Celis et al., 2004; Furuichi and Yone, 1981; Ince, 1979). Insulin increases trout capacity to lower glycemia [$R_d - R_a$] by decreasing both hepatic glucose production and glucose disposal (Fig. 2.3). Therefore, insulin could cause the decrease in glucose fluxes and the capacity to increase glycemia [$R_a - R_d$]. Unfortunately, fish insulin cannot be measured accurately (Moon, 2001). A radioimmunoassay was developed (Plisetskaya, 1998), but it also measures multiple pro-insulins and, therefore, overestimates true insulin concentration. Furthermore, the effects of pro-insulin has not been established. Overall, insulin is the most likely signal causing the decrease in glucose fluxes observed here after 4 h of glucagon administration.

Regulation of phosphoenolpyruvate carboxykinase

Multiple rounds of whole genome duplication events occurred during the evolutionary radiation of salmonids resulting in multiple isoforms of enzymes (Berthelot et al., 2014). This is the first study to show the regulation of three trout *Pepck* isoforms during the administration of glucagon (Fig. 3.5). The heavily researched cytosolic form of PEPCK (trout: *Pepck1*, mammal: PEPCK-C) is strongly stimulated in both trout (Fig. 3.5A) and mammals (Iynedjian and Salavert, 1984). The cytosolic form of *Pepck* is strongly associated with the regulation of gluconeogenesis in both fish and mammals (Méndez-Lucas et al., 2013; Mommsen, 1986; Suarez and Mommsen, 1987). Therefore, the strong upregulation of *pck1* supports an increase in R_a glucose. Less is known about the regulation of the mitochondrial form of PEPCK in fish (*Pepck2*) and mammals (PEPCK-M). Until recently it was thought that there was only one mitochondrial form in salmonids, but there are in fact two (*Pepck2a* and *Pepck2b*). Glucagon causes downregulation of *pck2a* but does not induce a response from *pck2b*. In mammals, PEPCK-M plays a role in potentiating PEPCK-C gluconeogenesis (Méndez-Lucas et al., 2013), but does not show a response to the glucoregulatory hormones: glucagon and insulin (Stark and Kibbey, 2014). The different regulation of mitochondrial forms between fish and mammals may reveal a divergence in function. Functional divergence occurs in three ways: neofunctionalization (a gene copy develops a new function), subfunctionalization (each gene retains a subset of the original ancestral gene), or conservation of function. Therefore, the differential regulation of mitochondrial *pck2a* may reflect neofunctionalization. Overall, minor increases in trout R_a glucose could be connected with the different regulation and function of *pck2* isoforms, and this hypothesis awaits rigorous functional testing in rainbow trout.

Regulation of glycogen phosphorylase

This is the first study to show that glucagon regulates the expression of two isoforms of glycogen phosphorylase differently (stimulation of *gpb*, and inhibition of *gpmuscle*) (Fig. 3.4). Although stimulation of *gpb* happens in muscle, the impact of this stimulation is relatively low when considering the transcript abundance in this tissue (Table 3.4). Also, *gpb* was measured in the liver and showed no response. Therefore, glucagon could have a tissue-specific (liver and muscle) regulation of *gpb*. On the other hand, the regulation of *gpmuscle* may have more impact on overall glycogen breakdown because it is highly expressed in muscle and lowly expressed in other tissues (Table 3.5). The inhibition of *gpmuscle* could indicate a counterregulatory response by insulin. In mammals, insulin strongly inhibits glycogen phosphorylase (Dimitriadis et al., 2011) but does not affect fish glycogen phosphorylase (Polakof et al., 2010). Insulin increases the expression of glycogen phosphorylase in the insulin impaired diabetic rat liver (Reynet et al., 1996), but no such measurements have been performed on fish. But, the experiments on diabetic rat liver may represent defective regulation because the liver is insulin impaired. Reynet et al. showed that insulin regulates glycogen phosphorylase at the transcriptional level. Therefore, insulin could also regulate glycogen phosphorylase transcripts in fish. Overall, glycogen phosphorylase isoforms have different transcript regulation in muscle, and *gpb* shows a tissue-specific regulation. Furthermore, the response of *gpmuscle* may suggest that insulin causes a counterregulatory response during the final 2 hours of the experiment.

Glucagon signalling pathway

Glucagon was unable to activate PKA substrates and FoxO1 in muscle and liver (Fig. 3.6). Glucagon has small glycogenolytic capabilities in muscle but mainly acts on the liver (Polakof et al., 2012). The lack of activation of the glucagon signalling pathway in muscle and liver may be associated with an overabundance of glucagon. The hormone possibly regulates the glucagon receptor concentration in fish hepatocytes (Navarro and Moon, 1994), like mammalian species (Horwitz and Gurd, 1988; Santos and Blazquez, 1982). *In vivo* studies in rats, report that high levels of glucagon cause downregulation of glucagon receptors (Dighe et al., 1984; Soman and Felig, 1978; Srikant et al., 1977). Overall, therefore, the lack of activation of the signalling cascade may be connected to downregulation of the glucagon receptors.

Conclusions and significance

This study is the first to provide measurements of glucose fluxes in rainbow trout during *in vivo* glucagon administration. It shows that trout progressively increase blood glucose concentration by causing an initial mismatch between R_a and R_d glucose that gradually disappears over the duration of the experiment (Figs. 3.1A and 3.2C). The observed hyperglycemia is probably due to an increase of R_a glucose via the stimulation of gluconeogenesis and glycogen phosphorylase, and a decrease of R_d glucose through inhibition of glycogen synthase and glycolysis. During the final 2 h of the experiment, both R_a and R_d glucose progressively decrease to values lower than baseline. The decrease of glucose fluxes may be a counterregulatory response modulated by insulin. Results also show that the 3 known Pepck isoforms of trout are regulated differently at

the transcript level (Fig. 3.4). The different regulation of *pck2a* may be connected to the minor increase of trout R_a glucose and possibly a new function of the mitochondrial form. Furthermore, glycogen phosphorylase isoform transcripts are regulated differently within muscle, and between tissues: no effect on liver *gpb* and upregulation of muscle *gpb* by glucagon (Fig. 3.5). This suggests that glucagon has isoform and tissue specific regulation of glycogen phosphorylases and the regulation of *gpmuscle* may be associated to insulin causing a counterregulatory response. I conclude that the inability to tightly control glycemia displayed by rainbow trout can partially be explained by the weak response of glucose fluxes to glucagon.

Table 3.1: Mean physical characteristics and hematocrit of the 2 groups of catheterized rainbow trout used (i) for *in vivo* measurements of glucose kinetics or (ii) for tissue measurements of glucagon signalling proteins and gene expression of enzymes. Values are means \pm s.e.m (sample sizes in parentheses)

	Glucose kinetics experiments (8)	Protein and gene experiments (14)
Body mass (g)	350 ± 16	344.4 ± 15
Body length (cm)	31.4 ± 0.4	31.7 ± 0.4
Hematocrit (%)	19.5 ± 0.3	19.5 ± 0.4

Table 3.2: Initial (baseline) and final values after 4 h of glucagon administration for various parameters of glucose metabolism in rainbow trout. Values are means \pm s.e.m (N=8). Glucose turnover rate (R_t) was obtained with the steady state equation, whereas the rates of appearance (R_a) and disposal (R_d) were obtained with the non-steady state equations of Steele (Steele, 1959). The effects of glucagon are indicated as ** P<0.001 (paired t-test).

	Baseline	Final (after 4 h of glucagon infusion)
Glucose ($\mu\text{mol ml}^{-1}$)	8.5 ± 0.4	$11.7 \pm 0.4^{**}$
R_t Glucose ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	11.5 ± 1.2	10.2 ± 0.9
R_a Glucose ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	10.6 ± 1.4	8.8 ± 1.3
R_d Glucose ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	10.6 ± 1.4	8.8 ± 1.2

Table 3.3: Primer sequences and annealing temperatures used for mRNA quantification by real-time RT-PCR

Target	Forward primer sequence (5'3')	Reverse primer sequence (3'5')	Annealing temperature [°C]	Reference
<i>pck1</i>	ACAGGGTGAGGCAGATGTAGG	CTAGTCTGTGGAGGTCTAAGGGC	55	(Marandel et al., 2015)
<i>pck2a</i>	ACAATGAGATGATGTGACTGCA	TGCTCCATCACCTACAACCT	56	(Marandel et al., 2015)
<i>pck2b</i>	AGTAGGAGCAGGGACAGGAT	CCGTTCAGCAAAGGTTAGGC	59	Designed
<i>g6pase</i>	TAGCCATCATGCTGACCAAG	CAGAAGAACGCCACAGAGT	55	(Panserat et al., 2009)
<i>fbpase</i>	GCTGGACCCTTCCATCGG	CGACATAACGCCACCATAGG	60	(Panserat et al., 2009)
<i>glut4</i>	GGCGATCGTCACAGGGATTC	AGCCTCCCAAGCCGCTCT T	57	(Panserat et al., 2009)
<i>glut2</i>	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	60	Designed
<i>gpmuscle</i>	CCCGGCTACAGGAACAACAT	ACAGCCTGAATGTAGCCACC	55	Designed
<i>gpb</i>	GTGATCCCTGCAGCTGACTT	TCCTCTACCCTCATGCCGAA	59	Designed
<i>glut1bb</i>	GTGATCCCTGCAGCTGACTT	AGGACATCCATGGCAGCTTG	57	(Liu et al., 2017)
<i>pfk</i>	CGTAGGCATGGTGGGTTCTA	AGCCACAGTGTCTACCCATC	59	Designed

Table 3.4: Glycogen phosphorylase b transcript numbers in various tissues via PhyloFish (Pasquier et al., 2016). Phylofish provides a comprehensive gene repertoire from de novo assembled RNA-sequence data in a large number of teleost species.

Tissue	Transcript counts
Hypophysis	265
Brain	340
Stomach	633
White muscle	51
Red muscle	71
Gills	289
Heart	51
Intestine	324
Liver	576
Ovary	469
Bones	125
Skin	210
Spleen	270
Head kidney	1026
Kidney	495

Table 3.5: Glycogen phosphorylase muscle transcript numbers in various tissues via PhyloFish (Pasquier et al., 2016). Phylofish provides a comprehensive gene repertoire from de novo assembled RNA-sequence data in a large number of teleost species.

Tissue	Transcript counts
Hypophysis	212
Brain	96
Stomach	170
White muscle	210747
Red muscle	66546
Gills	30
Heart	39715
Intestine	49
Liver	27
Ovary	35
Bones	90848
Skin	3184
Spleen	28
Head kidney	59
Kidney	28

Fig. 3.1. Effects of glucagon on plasma glucose concentration (panel A) and glucose specific activity (panel B). Values are means \pm s.e.m (N=8). Means significantly different from baseline are indicated by * ($P<0.05$; one-way RM ANOVA).

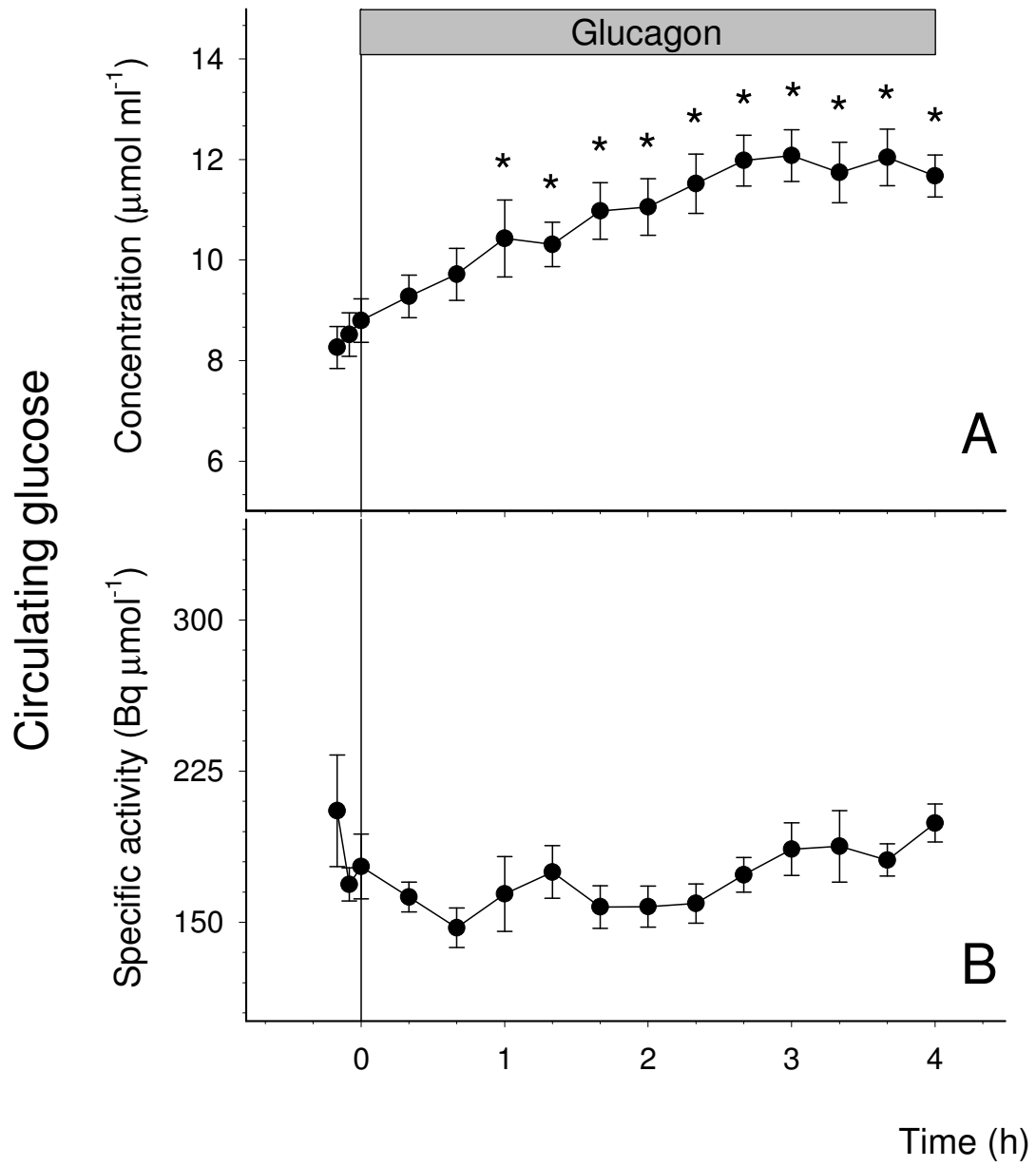


Fig. 3.2. Effects of glucagon on the glucose fluxes of rainbow trout. Fluxes were either calculated with the steady-state equation [turnover rate (R_t), panel A] or the nonsteady-state equations of Steele [glucose disposal (R_d) and hepatic glucose production (R_a), panel B] (Steele, 1959). Effects of glucagon on the capacity to increase glycemia [R_a glucose – R_d glucose, panel C] compared to baseline of 0. Values are means \pm s.e.m. (N=8). Means significantly different from baseline are indicated by * ($P<0.05$; one-way RM ANOVA).

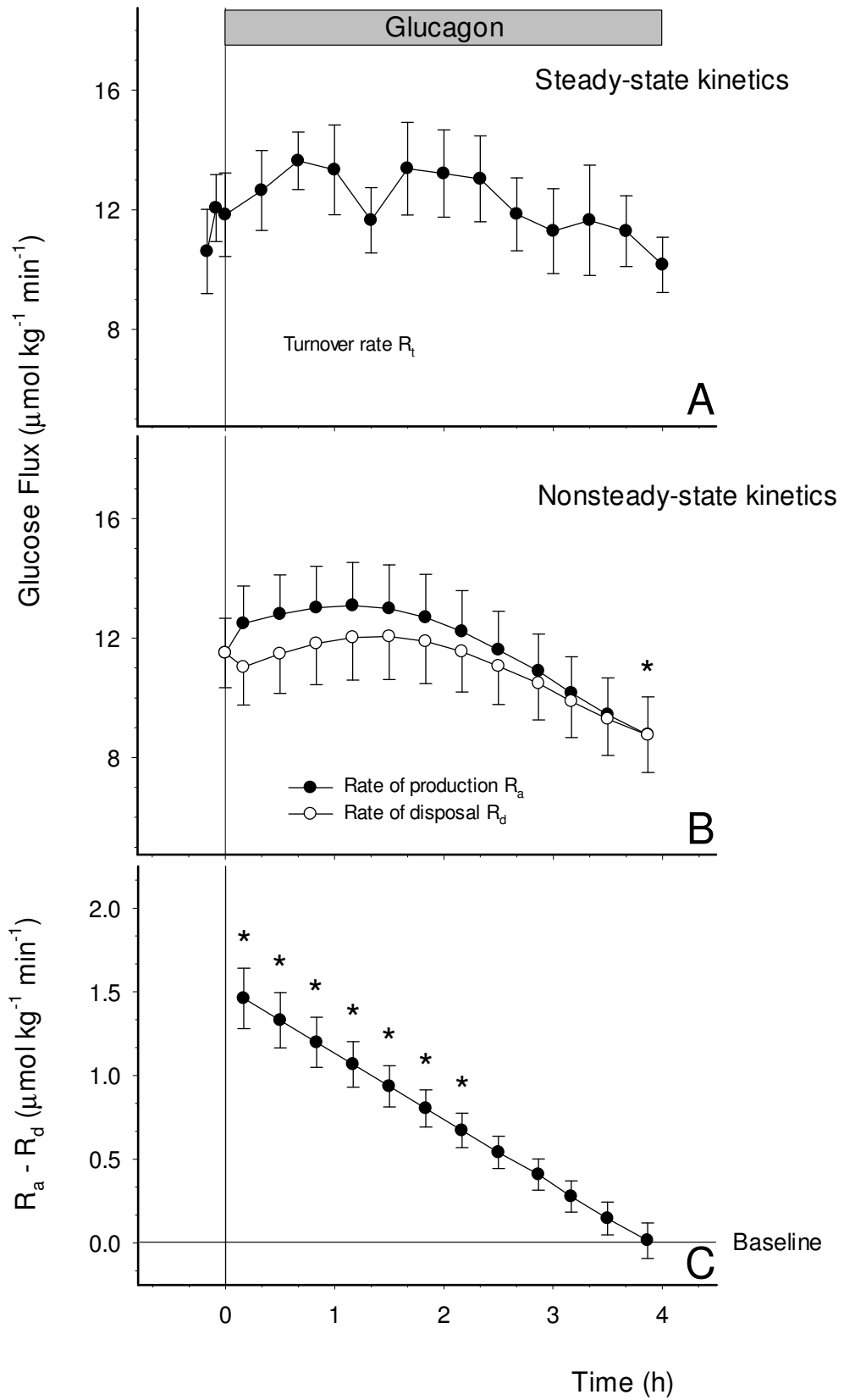


Fig. 3.3. Relative effects of glucagon on muscle mRNA transcript abundance of *glycogen phosphorylase b (gpb)* (panel A), *phosphofructokinase (pfk)* (panel B), *glycogen phosphorylase muscle (gpmuscle)* (panel C), *glucose transporter 4 (glut4)* (panel D), and *glucose transporter 1 (glut1)* (panel E). Values are means \pm s.e.m. (N=7 for each group). Means significantly different from control are indicated by * ($P<0.05$; Mann-Whitney Rank Sum Test) ** ($P<0.01$; unpaired t-test).

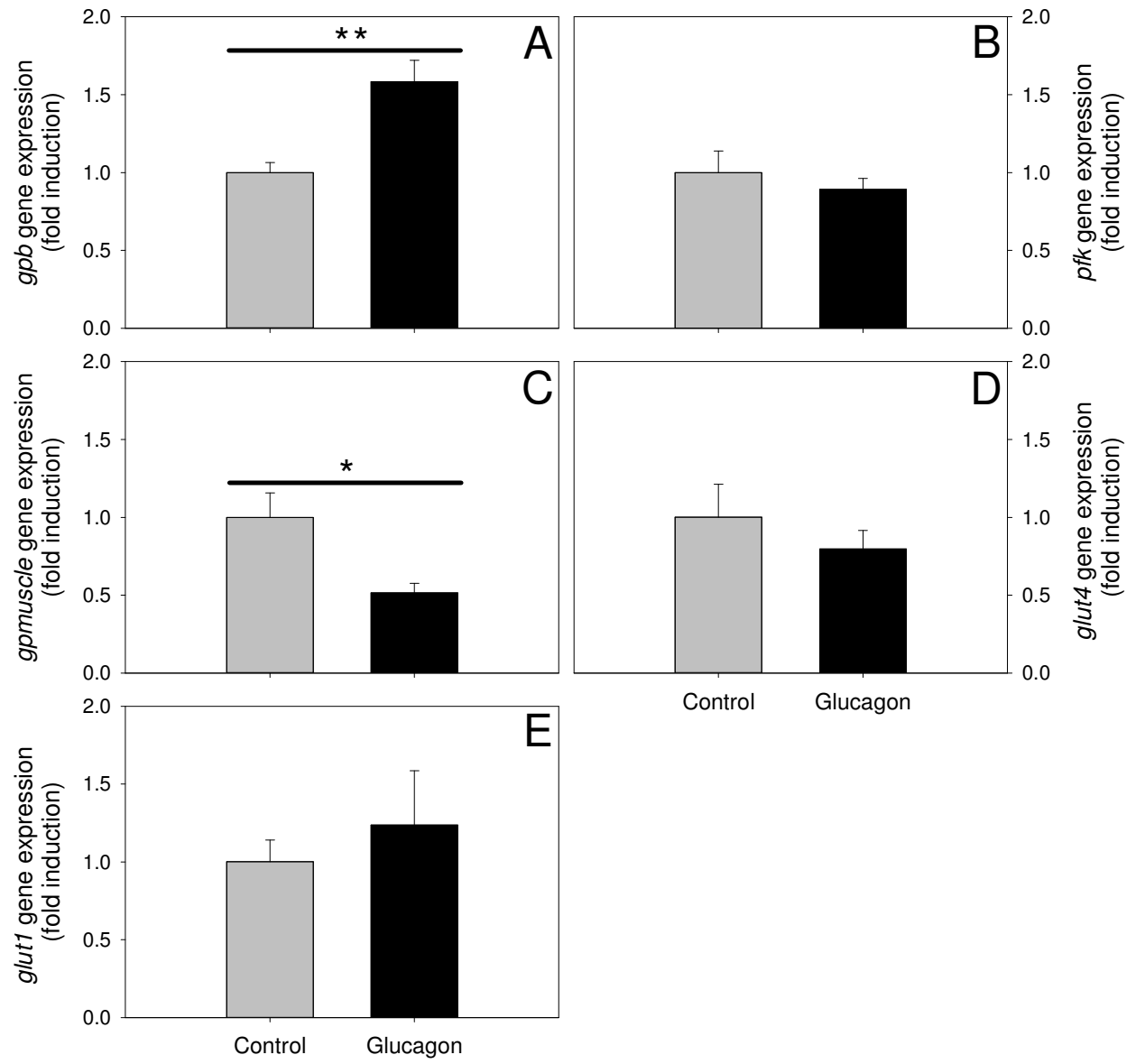


Fig. 3.4. Relative effects of glucagon on liver mRNA transcript abundance of *phosphoenolpyruvate carboxykinase 1 (pck1)* (panel A), *glucose-6-phosphatase (g6pase)* (panel B), *phosphoenolpyruvate carboxykinase 2a (pck2a)* (panel C), *glucose transporter 2 (glut2)* (panel D), *phosphoenolpyruvate carboxykinase 2b (pck2b)* (panel E) and *fructose 1,6-bisphosphatase (fbpase)* (panel F). Values are means \pm s.e.m. (N=7 for each group). Means significantly different from control are indicated by * ($P<0.05$; unpaired t-test) ** ($P<0.001$; Mann-Whitney Rank Sum Test).

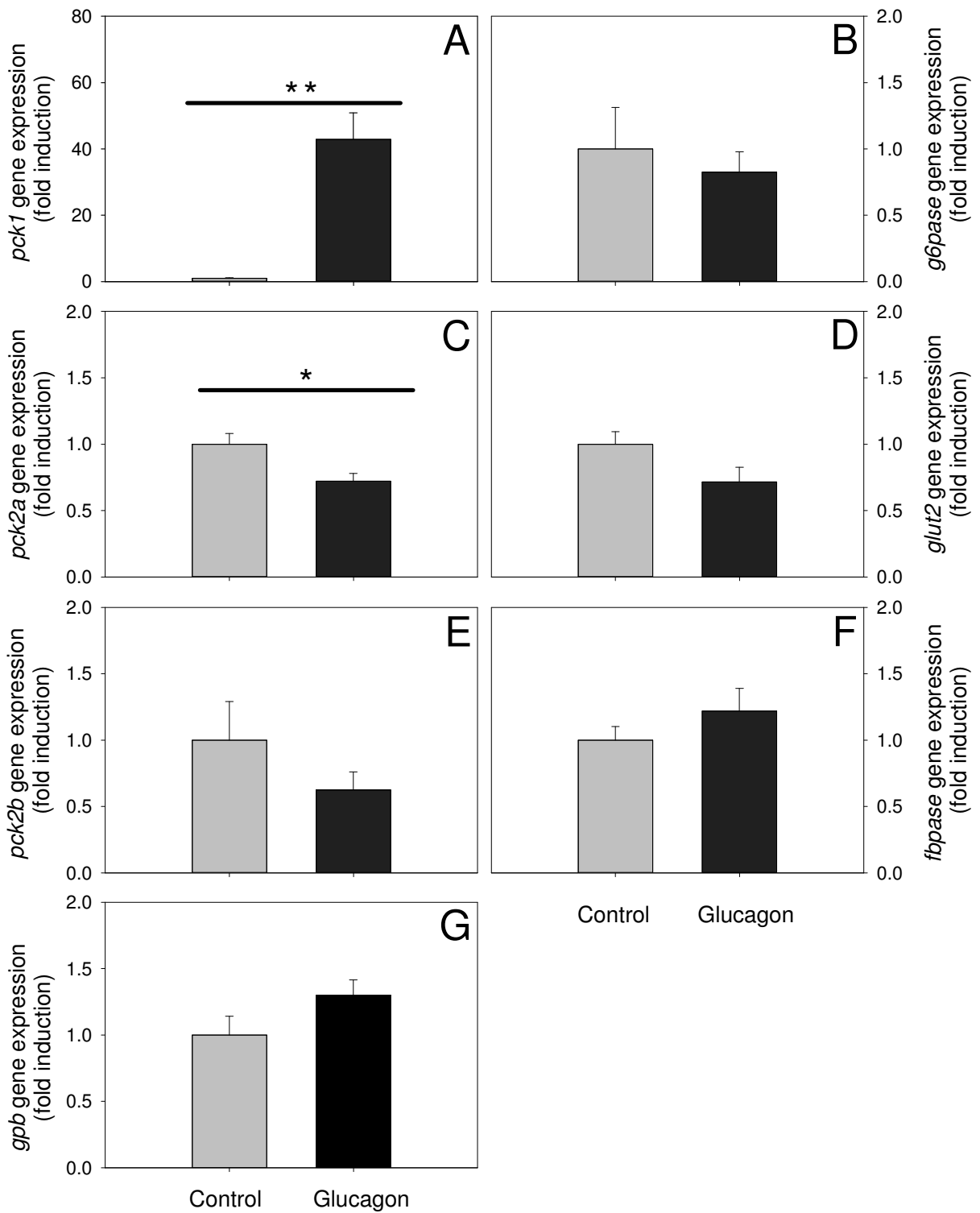


Fig. 3.5. Circulating glucagon levels in rainbow trout during glucagon administration.

Values are means \pm s.e.m. (N=8). Means significantly different from baseline are indicated by * ($P < 0.05$; one-way RM ANOVA).

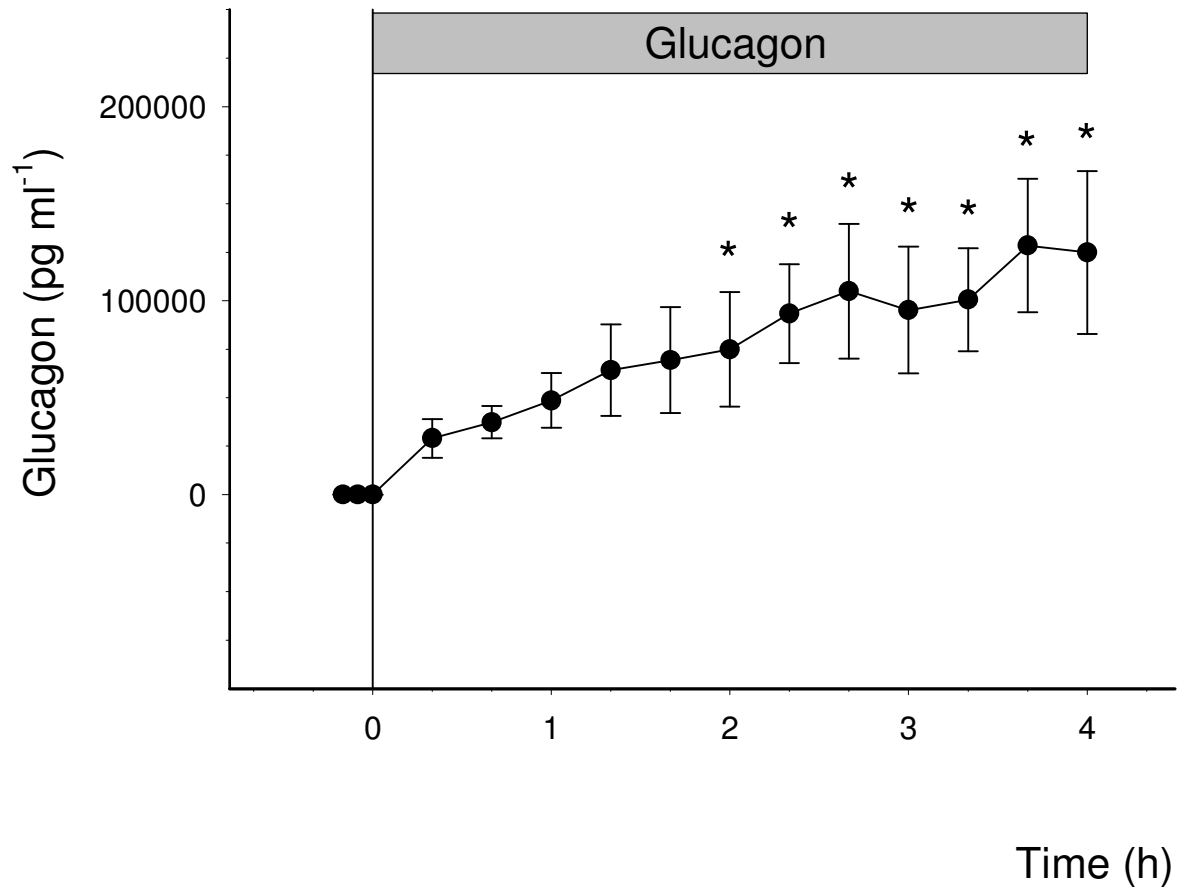
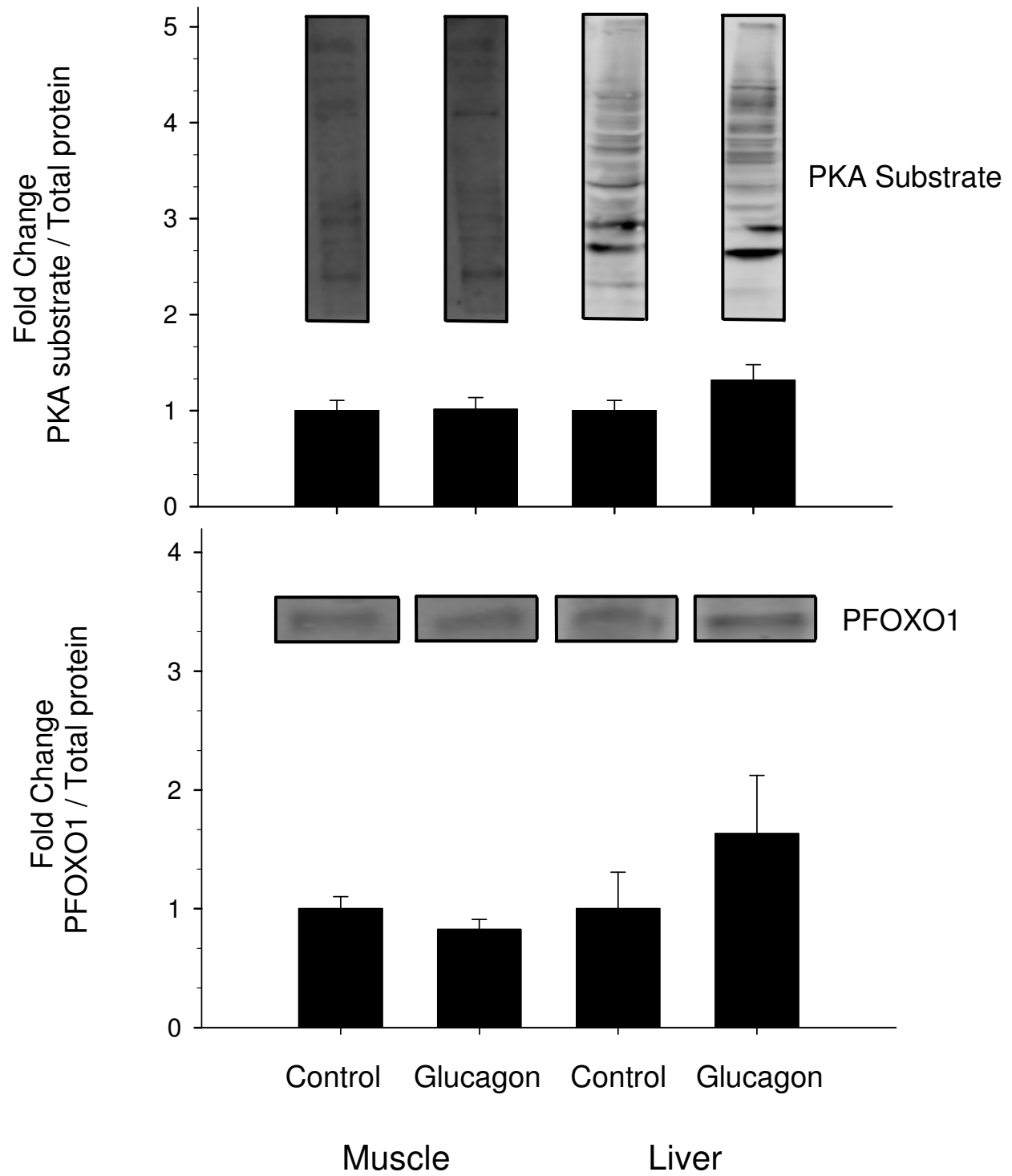


Fig. 3.6. Relative effects of glucagon on the levels of key signalling proteins. For each mean, western blots are given for the phosphorylated protein(s). Values are means \pm s.e.m. (N=6 for each group).



Chapter 4
General Conclusions

Thesis Overview

The main goal of this thesis was to assess the capacity of rainbow trout for glucoregulation by measuring glucose fluxes during the administration of the key glucoregulatory hormones insulin and glucagon. Rainbow trout have been considered glucose intolerant based on results from glucose tolerance tests (Legate et al., 2001). Choi and Weber provided evidence suggesting that trout may be better glucoregulators than previously thought (Choi and Weber, 2015). In their study, they infused rainbow trout with glucose at twice the resting rate of endogenous glucose production. This treatment caused endogenous R_a glucose to be completely suppressed and R_d glucose to be stimulated by 160%. The main signal thought to be responsible for these changes in glucose fluxes was insulin. However, the effects of hormones on glucose kinetics had never been investigated in rainbow trout except for epinephrine (Weber and Shanghavi, 2000). In the first part of my thesis, (Chapter 2), I have infused insulin into the dorsal aorta to evaluate whether the rates of hepatic glucose production (R_a glucose) and disposal (R_d glucose) were affected by this hormone. In view of what is currently known about the effects of insulin on trout glucose metabolism (Caruso and Sheridan, 2011; Polakof et al., 2012), I had anticipated that trout would respond to insulin like mammals.

Contrary to expectations, trout responded very differently to insulin. They showed a decrease in R_d glucose (-34%) while mammals are known to show a substantial increase (+304%; (Lucidi et al., 2010)). Also, R_a glucose decreased in trout (-38%), but, not as strongly as in mammals (-99%; (Lucidi et al., 2010)). Concurrently, I determined that insulin activates the signalling proteins Akt and S6 in muscle and increases

glucagon release in the circulation. After characterizing the effects of insulin on glucose kinetics, I investigated the potential role of glucagon on trout glucoregulation. In Chapter 3, glucagon was infused to measure the effects of this hormone on glucose kinetics, glucagon signalling cascade activation and gene expression of key enzymes of glucose metabolism. Previous information suggested that glucagon should cause the same response in trout as in mammals (Plisetskaya and Mommsen, 1996).

In mammals, glucagon increases R_a glucose very strongly, and weakly inhibits (Lins et al., 1983) or does not affect (Hinshaw et al., 2015) R_d glucose. Results showed that trout R_a and R_d do not respond to glucagon for over 3 hours, but eventually cause a significant decrease in both fluxes at 4 hours. Results also showed that PKA substrates and FoxO1 are not activated by glucagon. Finally, glucagon caused significant changes in the muscle mRNA abundance of *gpb* (increase) and *gpmuscle* (decrease). In liver, significant changes in the mRNA of *pck1* (increase) and *pck2a* (decrease) were measured.

General Discussion

Hormonal regulation of glucose disposal

This research is the first to quantify glucose fluxes during the administration of insulin and glucagon (Fig. 4.1A). I anticipated that R_d glucose would respond to these hormones similarly in trout than in mammals. Unexpectedly, insulin caused a decrease in glucose disposal instead of stimulating it like in mammals. These divergent responses may be associated to the regulation of gluco / hexokinase transcripts via SREBP-1.

Both animals show an increase in SREBP-1 after insulin administration, but hexokinases are downregulated in trout and upregulated in mammals (Panserat et al., 2014; Vogt et al., 1998). The downregulation of these enzymes slows down phosphorylation rates of glucose entering the cell. In turn, intracellular free glucose increases and this increase reduces the transmembrane concentration gradient driving inward glucose transport via GLUTs. In addition, trout responded to glucagon in a similar way to mammals receiving glucagon and somatostatin (Lins et al., 1983), whereby R_d glucose slightly decreased but was mostly unaffected. This response is unusual because two parameters (glycolysis and glycogen synthesis) that affect the rate of glucose disposal have been shown to be inhibited in trout and mammals. After glucagon administration, both animals decrease the activity of glycogen synthase and the glycolytic enzymes, pyruvate kinase (PK) and phosphofructokinase (PFK) (Foster et al., 1989; Jiang and Zhang, 2003; Murat and Plisetskaya, 1977; Petersen et al., 1987). However, other enzymes involved in glycolysis and glycogen synthesis like the hexokinases may play a more significant role in the trout R_d glucose response (Panserat et al., 2014; Wasserman et al., 2011; Zhou et al., 2018). Overall, when compared to mammals, trout R_d glucose responds in the opposite way to insulin, but similarly to glucagon.

Hormonal regulation of glucose production

This thesis quantifies the effects of insulin and glucagon on trout R_a glucose (Fig. 4.1B). Trout hepatic glucose production shows a weaker response than mammals when treated with insulin or glucagon. Insulin inhibited R_a glucose in trout (-43%), but

mammals almost completely inhibit it (-99%; (Lucidi et al., 2010)). The weak inhibition of trout R_a glucose may be linked to changes in the rates of glycogen breakdown, gluconeogenesis or both. The rate of glycogen synthesis or breakdown is determined by the activity ratio between glycogen phosphorylase and glycogen synthase (Pereira et al., 1995). These enzymes seem to be unresponsive in trout (Polakof et al., 2010c), but, in mammals, insulin strongly inhibits glycogen phosphorylase and stimulates glycogen synthase (Dimitriadis et al., 2011; Shrayyef and Gerich, 2010). In addition, the weak response of trout R_a glucose to insulin could be associated with differences in the regulation of gluconeogenesis. Previous studies report that key gluconeogenic enzymes like Pepck, FBPase, and G6Pase are downregulated in trout and mammals (Polakof et al., 2010c; Saltiel, 2015). However, trout have multiple forms of these enzymes from gene duplication events in their evolutionary history, and it is suggested that these isoforms may be regulated differently (Marandel et al., 2015). In addition, trout R_a glucose shows a weak response when glucagon is infused. The weak stimulation of R_a glucose may be connected to gluconeogenesis and glycogen breakdown. The results show that Pepck is the only gluconeogenic enzyme to respond in trout. The different transcriptional regulation of the Pepck isoforms (*pck1*=stimulation, *pck2a*=inhibition, *pck2b*=no response) may explain why there is a weak response of R_a glucose in trout. Also, this supports the notion that gluconeogenic isoforms are differentially regulated as reported by Marandel et al. (Marandel et al., 2015). Furthermore, the little stimulation of R_a glucose in trout may also be linked to the weak stimulation of glycogen breakdown. During glucagon treatment, glycogen phosphorylase activity is stimulated by ~50% (Puviani et al., 1990) in trout, and by ~160% in mammals (Malbon et al., 1978),

therefore, lowering the capacity for glycogen breakdown. Overall, trout R_a glucose seems to show a limited response to insulin and glucagon, when compared to mammals.

Counterregulatory response

I was able to show that there is a counterregulatory response during the administration of insulin and possibly during glucagon treatment. The counterregulatory response to insulin occurred when glucagon concentration was increased at 3 hours. Glucosensing neurons, as well as A-cells in the Brockmann bodies, detect the changing blood glucose levels in fish (Plisetskaya and Mommsen, 1996). Once glycemia reached ~5 mM at time 2.67 hours (Fig. 2.2), the increase in glucagon concentration was detected. After the release of glucagon, the glucose fluxes begin to plateau. It is possible that other hormones may be present in trout to counteract insulin (catecholamines, growth hormone, glucagon-like-peptide, among others). However, no such measurements could be made because of the limited amount of plasma available. Additionally, a counterregulatory response to glucagon may have occurred within the 4 hour experiment. Results show over the final 2 hours blood glucose concentration stabilizes, glucose fluxes decrease progressively below baseline, and the capacity to raise glycemia [$R_a - R_d$] approaches zero. These results suggest that insulin is causing a counterregulatory response. Insulin secretion occurs when fish experience hyperglycemia (Blasco et al., 2001; de Celis et al., 2004; Furuichi and Yone, 1981; Ince, 1979). Insulin decreases R_a glucose slightly more than R_d glucose which increases the capacity to lower glycemia in trout. However, fish insulin cannot be measured accurately

and therefore, was not quantified. In conclusion, rainbow trout increase circulating glucagon levels to counteract the effects of insulin and possibly show a counterregulatory response during glucagon infusion.

Conclusions

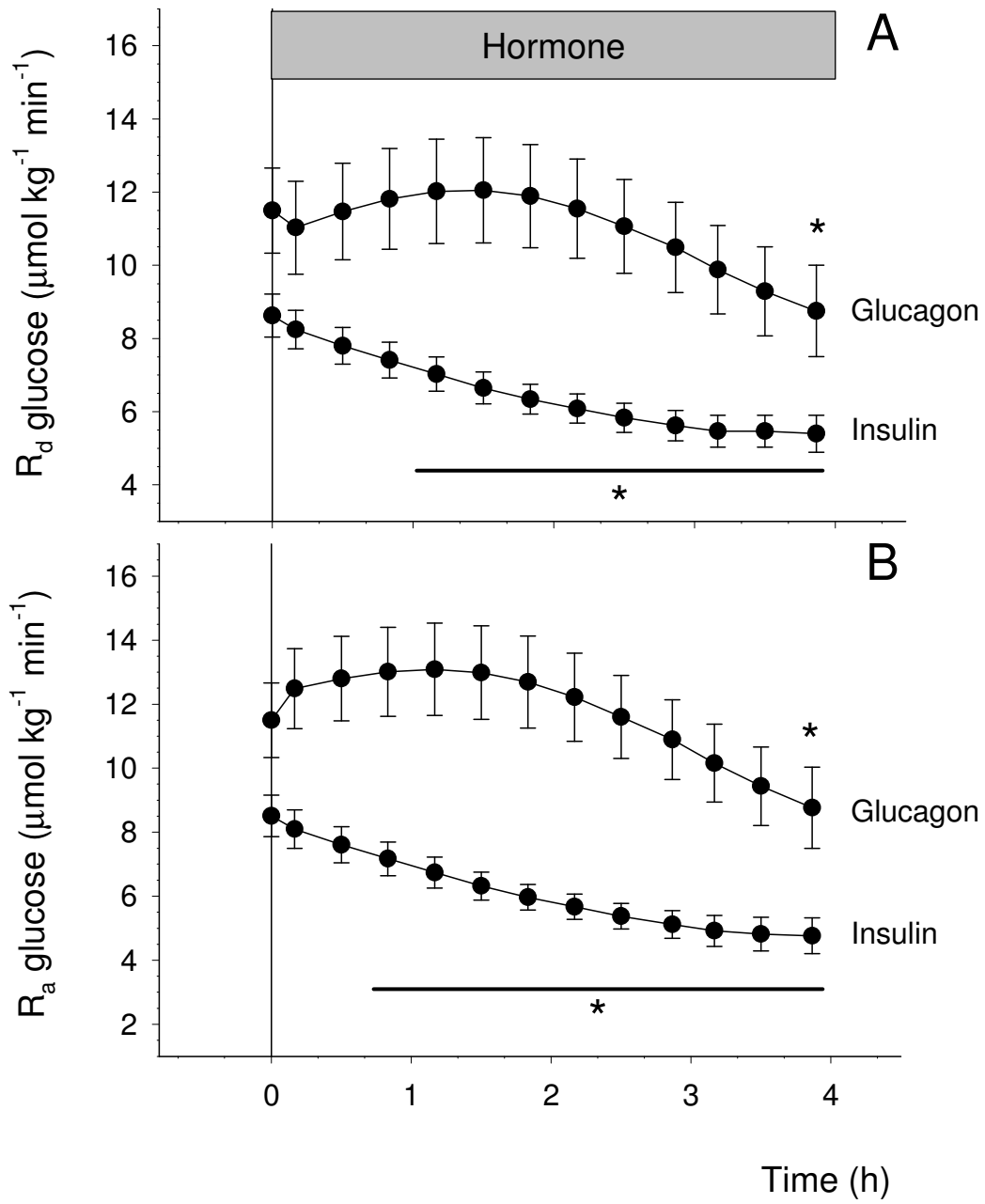
This thesis shows the effects of insulin and glucagon on glucose fluxes in rainbow trout. Both hormones have different effects on trout glucose fluxes than on mammal fluxes. During insulin administration, trout inhibited R_a glucose and unexpectedly decreased R_d glucose. The difference between R_d and R_a remained extremely small compared to what is known for mammals (Fig. 2.4). The unexpected inhibition of R_d and the small difference between the glucose fluxes helps to explain glucose intolerance in trout. During glucagon administration, trout displayed a weak response by not changing glucose fluxes from baseline until the final time point. However, an initial difference between R_a and R_d glucose caused an increase in glucose concentration (Fig. 3.2). The minor changes in response to glucagon may show that trout have a weak sensitivity to the hormone. In conclusion, rainbow trout are glucose intolerant compared to mammals because insulin and glucagon have either the opposite effect or much weaker, but similar effects than mammals.

Future directions

In the future, experiments should focus on the other hormones known to participate in the regulation of glucose metabolism as well as on understanding the

different mechanisms and actions of the glucoregulatory hormones that modulate glucose fluxes. Results from Chapter 2 show that insulin inhibits R_d glucose of trout but stimulates it in mammals, and this different response could be mediated through the regulation of glucokinase and hexokinase. It would be interesting to understand the regulation of these enzymes by SREBP-1 in trout. In Chapter 3, results showed that R_a and R_d glucose are weakly regulated by glucagon. Characterizing parts of the glucagon signalling pathway in fish will allow for a better understanding of what enzymes are regulated and will determine the role that these enzymes play in changing glucose fluxes. Also, other hormones control glucose metabolism like growth hormone, GLP-1, epinephrine, and cortisol. Characterizing the effects of these other hormonal signals individually (and possibly synergistically) will clarify the glucoregulatory capacity of rainbow trout. In addition, understanding the counterregulatory responses that control glucose levels will require specific assays of each of the hormones to determine their relative roles. Finally, the use of glucose-clamp experiments will be needed to clarify direct vs indirect effects of these endocrine signals.

Figure 4.1: Comparative effects of insulin and glucagon on the glucose fluxes of rainbow trout. Glucose fluxes were calculated using the nonsteady-state equations of Steele (Steele, 1959). Values are means \pm s.e.m. (Insulin; N=10, Glucagon; N=8). Means significantly different from baseline within each hormone treatment are indicated by * ($P<0.05$; one-way RM ANOVA).



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