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HEPATIC GLUCOSE PRODUCTION IN RAINBOW TROUT: EFFECTS OF SUSTAINED EXERCISE AND EPINEPHRINE

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ABSTRACT

The role of carbohydrate metabolism is not yet established in fish. Therefore, the goal of my thesis was to quantify the rate of hepatic glucose production (R_a glucose) during aerobic exercise in fish. During sustainable exercise, mammals strongly stimulate their rate of hepatic glucose production to replace the additional glucose consumed by locomotory muscles and this increase in R_a glucose may be regulated by circulating catecholamines. By analogy with mammals, I hypothesized that sub-maximal exercise in trout will increase hepatic glucose production above resting levels. Circulating catecholamines were also measured to establish their possible influence in stimulating R_a glucose. To test this hypothesis, I measured the rate of appearance of glucose in rainbow trout swimming for 3 h at 1.5 body lengths s^{-1}. Contrary to what I had hypothesized, sustained swimming caused R_a glucose to decline by 33% from 7.6 ± 2.1 to 5.1 ± 1.3 μmol kg^{-1} min^{-1} (N=7). At the end of 3 h exercise, circulating epinephrine (EPI) had also decreased by 30% suggesting that EPI might be partly responsible for controlling the decline in R_a glucose. Even 1 h after the end of exercise, hepatic glucose production had not returned to resting values. Surprisingly, glucose concentration was maintained constant throughout exercise by matching rates of hepatic glucose production and peripheral glucose utilization, even though trout are generally considered as poor glucoregulator. This study shows that circulating glucose is probably not an important oxidative fuel to support aerobic locomotion (Chapter 2).

Results from Chapter 2 indicated that circulating EPI could be a possible candidate for regulating R_a glucose during exercise. Therefore, I investigated the effects of sympathetic EPI on R_a glucose 1) by infusing exogenous EPI to increase plasma concentrations and 2) by blocking the effect of sympathetic EPI via β-adrenoreceptors with propranolol (PROP). I hypothesized that EPI will stimulate whereas PROP will depress hepatic glucose production. Elevated circulating EPI levels (~545 nmol l^{-1}) *in vivo* caused hyperglycemia by increasing R_a glucose from 6.57 ± 0.79 μmol kg^{-1} min^{-1} to 13.30 ± 1.78 μmol kg^{-1} min^{-1} (N=7) but EPI in trout does not decrease peripheral glucose utilization as observed in mammals. Infusion of PROP depressed R_a glucose by 46% from 7.65 ± 0.92 μmol kg^{-1} min^{-1} to 4.10 ± 0.56 μmol kg^{-1} min^{-1} (N=10) suggesting that basal levels of sympathetic EPI are partly responsible for maintaining resting R_a glucose. Glucose production was also quantified in isolated hepatocytes using the same group of fish. Here, I hypothesized that: a) *in vivo* and *in vitro* rates of hepatic glucose production will be similar, b) EPI will increase whereas PROP (in presence of EPI) will depress glucose release in isolated hepatocytes and c) the magnitude of the effects of EPI or PROP will not be similar *in vivo* and *in vitro* because indirect effects of EPI will only be present *in vivo*. The basal rate of hepatic glucose production *in vitro* was 3.13 ± 0.09 (N=22) μmol kg^{-1} min^{-1}, one-half of the *in vivo* rate of 7.31 ± 0.54 (N=20) μmol kg^{-1} min^{-1}. However, in the intact fish, circulating EPI (5 nmol l^{-1}) is present and even incubating isolated hepatocytes with 5 nmol l^{-1} EPI only elevates R_a glucose by 20%, one half the *in vivo* rate. Interestingly, high concentrations of EPI stimulated hepatic glucose production by two-fold *in vitro* to 5.92 ± 0.19 μmol kg^{-1} min^{-1}, a value similar to basal R_a glucose *in vivo*. These results show that EPI stimulates glucose

production both *in vivo* and *in vitro*, and that basal sympathetic EPI levels are required to maintain resting hepatic glucose production *in vivo* (chapter 3).

This thesis shows that EPI can play an important role in the regulation of hepatic glucose metabolism, and that basal sympathetic concentration of EPI may play a role in maintaining resting hepatic glucose production.

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CHAPTER 1

GENERAL INTRODUCTION

Carbohydrates are crucial to a cell for maintaining sufficient concentrations of ATP and in providing precursors for a variety of biosynthetic processes. An organism that undergoes stresses such as exercise, hypoxia or decreased dietary intake of carbohydrate must alter its carbohydrate catabolic patterns to change the rate of glucose production to maintain basal biosynthetic processes. Without a doubt, the regulation of glucose production exists in all organisms and is accomplished by hormonal and/or neuronal signals that modulate the activity of key enzymes in the catabolic pathways. For example, in mammals, catecholamines regulate the increase in glucose production during low-intensity exercise to provide extra ATPs for working muscles (Miles *et al.*, 1992). However, very little is known about the control of glucose production by catecholamines in rainbow trout (*Oncorhynchus mykiss*). Therefore, the primary objective of this thesis is to quantify hepatic glucose production (R_a glucose) during exercise and to determine if epinephrine (EPI) regulates R_a glucose in fish.

1. Glucose

1.1 Blood glucose

Although it has been established that carbohydrates act as an important energy source for mammals, their significance as an energy source for fish has not yet been determined. In mammals, regulation of glucose concentration is required due to the fact that glucose is the only nutrient that can be utilized by the brain, retina, nerve cells, erythrocytes and renal medulla (Guyton, 1981; Rawn, 1989). Also, if blood glucose is excessively high, it increases osmotic pressure in the extracellular fluid and can cause

cellular dehydration and osmotic diuresis by the kidneys (Guyton, 1981). Glucose homeostasis is of lower priority in teleosts, perhaps on account of their higher brain glycogen reserves (Plisetskaya, 1968). Fish are also known to tolerate high blood glucose load for very long periods without any of the side effects noted in mammals (Driedzic and Hochachka, 1978; Palmer and Ryman, 1972).

1.2 Sources of blood glucose

In the post-absorptive state, there are two organs that contribute to blood glucose concentrations. The first is the liver through the processes of glycogenolysis (breakdown of glycogen to free glucose) and gluconeogenesis (synthesis of glucose from non-carbohydrate source). The second organ is the kidney through gluconeogenesis. As in mammals, the kidney is only a minor contributor to total glucose production in fish since renal activities of key gluconeogenic enzymes are extremely low (Knox *et al.*, 1980; Suarez and Mommsen, 1987). Haman *et al.* (1997a) measured renal glucose production in hepatectomized trout, and estimated it to be approximately 10% of the total R_a glucose. However, this contribution by kidney is probably over estimated because the kidney may have been stimulated to compensate for the absence of the liver in hepatectomized animals. Because the kidney's contribution to gluconeogenesis is low, R_a glucose is often simply called hepatic glucose production in mammalian studies (Coggan *et al.*, 1990; Levy *et al.*, 1989; Vissing *et al.*, 1988; Wiersma *et al.*, 1993). Therefore, I will also use R_a glucose and hepatic glucose production interchangeably throughout this thesis.

2. Regulation of hepatic glucose production (neuronal and hormonal)

2.1 Hormonal Factors

In mammals, adrenal (catecholamines and corticoids) and pancreatic (insulin and glucagon) hormones are the key regulators of glucose metabolism. The pancreatic hormones play an important role in regulating R_a glucose under a variety of circumstances such as before and after a meal and during exercise whereas adrenal hormones regulate R_a glucose during acute stressful situations such as exercise and trauma.

2.1.1 Catecholamines

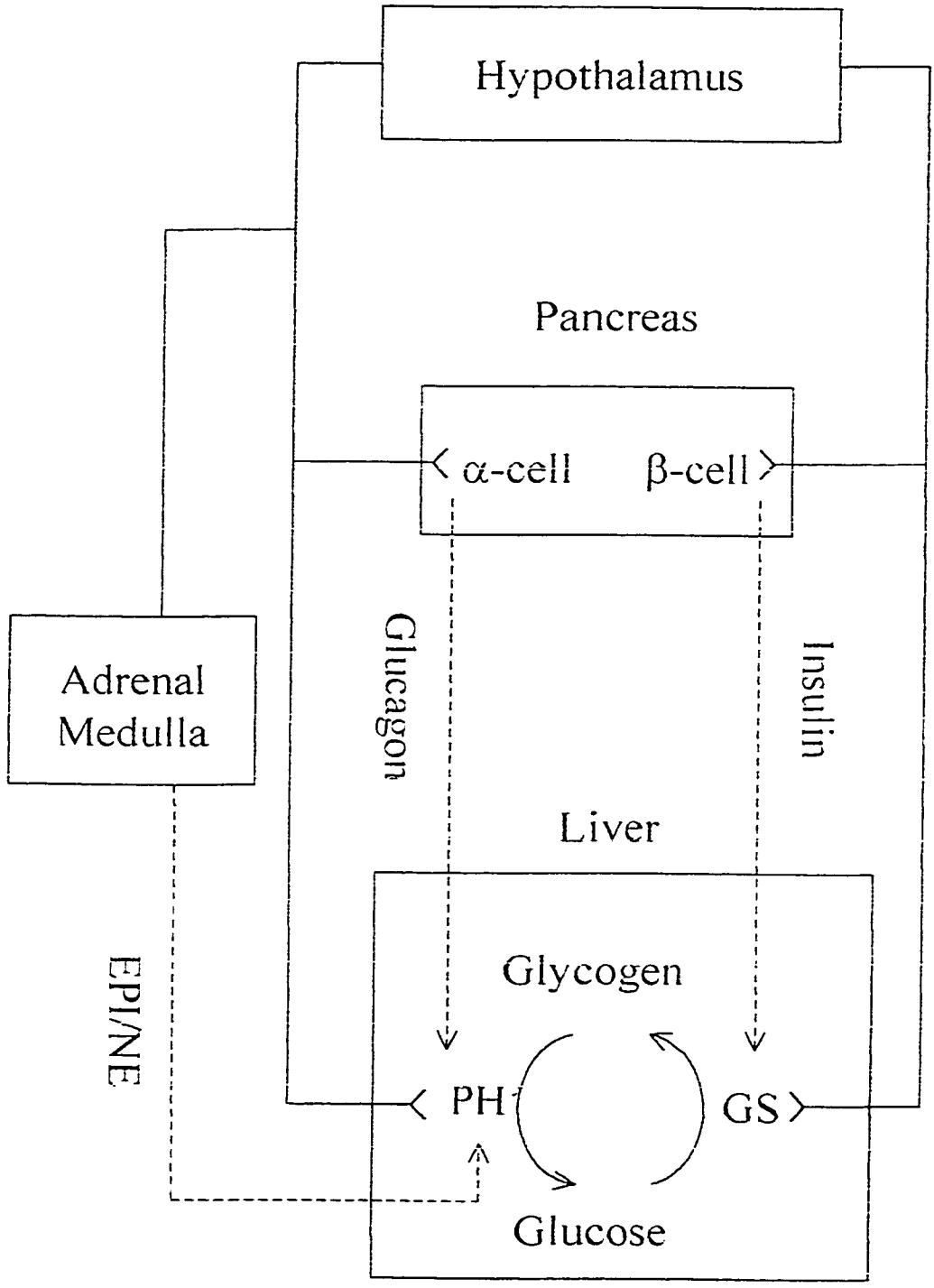
Epinephrine (EPI) and norepinephrine (NEPI) regulate glucose metabolism during periods of acute stress. In mammals, catecholamines levels are known to rise in response to increased central nervous system (CNS) input to the adrenal medulla. However, in fish catecholamines are released into the bloodstream by direct secretion from the chromaffin tissue and/or by diffusion from adrenergic nerve endings (combination of both is referred to as sympathetic catecholamines in this thesis) (Butler and Metcalfe, 1983). Upon release, they modulate cardiovascular and respiratory function in order to maintain adequate levels of oxygen in blood and therefore, a sufficient supply to the tissues (Reid *et al.*, 1998). In addition, they act on other receptor sites such as the liver to mobilize energy stores to provide for the increased energy demands that often accompany stress (Perry and Wood, 1989; Randall and Perry, 1992). In mammals, it has been established

that EPI promotes hepatic glucose production via stimulation of glycogenolysis and gluconeogenesis. However, in an intact fish, hepatic glucose production has never been measured during exogenous infusion of EPI. Presently, *in vitro* studies using isolated hepatocytes or liver slices incubated with EPI which have reported a rise in glucose production due to an increase in the rate of glycogenolysis and gluconeogenesis (Brighenti *et al.*, 1987; Fabbri *et al.*, 1995; Mommsen, 1986; Morata *et al.*, 1982; Suarez and Mommsen, 1987; Walton and Cowey, 1979). Wright *et al.* (1989) suggested that the rise in hepatic glucose production may be due to a 3- to 5-fold increase in the activity of glycogenolytic and gluconeogenetic enzymes. In mammals, EPI and NEPI are also known to indirectly regulate glucose metabolism by binding to α– and β-islets of the pancreas (Pagliassotti *et al.*, 1994) as depicted in Figure 1.1.

2.1.2 Pancreatic Hormones

Insulin and glucagon are released by β- and α- cells of the pancreas, respectively. Insulin is secreted after a meal or during hyperglycemia to lower blood glucose to its normal level (Cortright and Dohm, 1997; Pagliassotti *et al.*, 1994). Insulin brings plasma glucose to baseline concentrations by 1) stimulating the transport of glucose from the blood into cells (particularly muscle), 2) suppressing hepatic glucose output by decreasing the rate of gluconeogenesis and 3) by storing glucose as glycogen (Pagliassotti *et al.*, 1994). Insulin also stimulates the transport of amino acids into cells for the synthesis of proteins and stimulates the conversion of glucose and other nutrients into fatty acids and triacylglycerol (Holness *et al.*, 1996; Lehninger *et al.*, 1993; Pagliassotti *et al.*, 1994).

Figure 1.1 Direct neuronal and neuronal-hormonal mechanisms involved in hypothalamic control of liver glycogen metabolism of mammals. Neuronal pathways are indicated by solid lines and hormonal pathways by dashed lines. PH, glycogen phosphorylase; GS, glycogen synthetase. Revised from (Shimazu, 1987).


Hypothalamus
Pancreas
α-cell
β-cell
Glucagon
Insulin
Adrenal Medulla
Liver
EPI/NE
Glycogen
PH
GS
Glucose

Even though injecting insulin in fish causes hypoglycemia, glucose does not stimulate insulin secretion in this group of vertebrates (Ince, 1983). Amino acids, particularly arginine and lysine, are actually insulin secretagogues in trout (Mommsen and Plisetskaya, 1991) and eel (Ince and Thorpe, 1977). Furthermore, Gutierrez and Plisetskaya (1990) have shown that insulin binds to hepatocyte membranes of rainbow trout. Therefore, these findings suggest that insulin release would depress hepatic glucose metabolism in fish, although insulin is not secreted in response to hyperglycemia.

Glucagon is secreted during hypoglycemia, fasting and exercise when it acts to accelerate glycogenolysis in the liver to increase glucose output. Glucagon also affects gluconeogenesis by increasing the ability of the liver to take up gluconeogenic amino acids that can activate the gluconeogenic pathway (Pagliassotti *et al.*, 1994). In teleosts, the administration of mammalian glucagon generally elicits a hyperglycemic response (Chan and Woo, 1978; Ince and Thorpe, 1977; Moon, 1998; Thorpe and Ince, 1974). Glucagon also stimulates gluconeogenesis in eel, trout and carp by increasing plasma amino acid uptake by the liver that probably originated from muscles (Chan and Woo, 1978; Walton and Cowey, 1979). However, the effects of insulin and glucagon on R_a glucose have never been measured in an intact fish.

2.2 Neuronal Factors

Neuronal control is another mechanism by which mammals regulate their glucose production and uptake. Niijima (1983) was the first to characterize the existence of glucoreceptors in the mammalian liver. These glucoreceptors monitor glucose levels in portal venous blood and if glucose concentration increases in the portal vein, the

glucoreceptors would decrease its signalling rate to CNS. Glucoreceptors send signals to the CNS through the vagus nerve and the hyothalamus transmits signals to the liver via efferent pathways of both sympathetic and parasympathetic divisions of the autonomic nervous system (Pagliassotti *et al.*, 1994; Shimazu, 1987). Stimulation of the sympathetic nervous system leads to activation of many catabolic processes including glycogenolysis, gluconeogenesis and an increase in hepatic blood flow that results in increase of glucose output into the circulation. Conversely, stimulation of the parasympathetic nervous system enhances glucose uptake for glycogenesis and inhibits gluconeogenesis. This results in a decreased in R_a glucose. Neural factors are also able to control pancreatic hormones and catecholamines to achieve the same metabolic response (see Fig. 1.1).

In teleost fish, it has not yet been determined to what extent these different nerve fibres regulate hepatic glucose production. So far, it is known that the sympathetic preganglionic fibres exit from many cell bodies in the spinal cord to form a longitudinal chain that connects to the liver (Satchell, 1991).

3. Hepatic glucose production in fish

Fish must constantly adjust their rates of glucose production to co-ordinate the biochemical processes involved in maintenance, growth, reproduction, locomotion and stress (Weber and Zwingelstein, 1995). Therefore, understanding the regulation of glucose flux in fish is essential. Increased blood glucose concentration is used as an index of biochemical stress in fish (Weber and Zwingelstein, 1995). However, hyperglycemia is the only indication of an imbalance between release and uptake of glucose and tells you little about changes in R_a glucose, because flux and concentration do not necessarily change in parallel (Wolfe, 1992).

Haman and colleagues (Haman *et al.*, 1997a; Haman and Weber, 1996) developed and validated a continuous infusion technique for use in rainbow trout, allowing the measurement of R_a glucose in non-steady states. These authors were then able to quantify glucose turnover in non-steady states such as hypoxia and acute cold exposure. They (Haman *et al.*, 1997b) were the first to show a transient increase in hepatic glucose production under hypoxia and a decrease with a rapid drop in temperature. Only one other study has quantified glucose flux using the continuous infusion technique in American eels (Cornish and Moon, 1985) and provided evidence that eels have an enhanced ability to utilize carbohydrates. All other published R_a glucose measurements have been carried out using the bolus injection technique (Andersen *et al.*, 1991; Bever *et al.*, 1981; Garin *et al.*, 1987; Machado *et al.*, 1989). Unfortunately, this method is somewhat less reliable and, therefore, some of the rates reported may be questionable (Haman *et al.*, 1997a).

3.1 Exercise

Glucose kinetics have been quantified in resting fish including rainbow trout, American eel and sea bass, they have never been quantified during active swimming. So far, most studies have focused on exhaustive exercise since carbohydrate utilization is evident during anaerobic metabolism (Moyes and West, 1995). However, wild salmonids spend most of their time swimming at submaximal velocities (Beamish, 1978; Quinn, 1988; Weihs, 1973). The limited data available on aerobic swimming suggest that the contribution of carbohydrates is low while lipid oxidation dominates (Driedzic and Hochachka, 1978; Moyes and West, 1995), but these findings do not help predict changes in R_a glucose. Recent results from Kieffer *et al.* (1998) showed that strenuous swimming causes a significant increase in total carbohydrate use in juvenile trout, but with their experimental approach, the respective contributions of muscle glycogen and circulating glucose cannot be separated. Furthermore, Parkhouse *et al.* (1988) showed that liver glycogen values do not change during sustained swimming, whereas red muscle glycogen content decreases significantly. This suggests that circulating glucose may not be an important fuel to support prolonged swimming in fish.

A great deal of research has been done on mammals exercising at low velocities (Coggan *et al.*, 1995; Weber *et al.*, 1990; Weber *et al.*, 1996). These studies provide detailed knowledge about the mechanisms by which exercise affects the rate of hepatic glucose production. During sub-maximal exercise, liver is apparently the sole source of glucose entry into the circulation, as no significant glucose release is detected from the kidneys (Wahren *et al.*, 1971). During exercise of moderate intensity and duration, plasma glucose concentration remains constant, increases, or decreases depending on the

species or the duration of exercise. Exercise intensity of 40% $\dot{M}O_2$ max stimulates R_a glucose by 2- to 4-fold to replace the additional glucose consumed by locomotory muscles (Brooks and Donovan, 1983; Weber *et al.*, 1990; Weber *et al.*, 1996).

During the early phase of exercise, increased R_a glucose is predominantly due to an increase in glycogenolysis (Ahlborg and Felig, 1982; Ahlborg *et al.*, 1974; Wahren *et al.*, 1971). Coggan (1991) showed that human hepatic glycogen concentration is reduced by roughly one-half after only 1 hour of moderate exercise. The contribution of gluconeogenesis to R_a glucose remains at resting level for the first 35 to 50 minutes (Stanley *et al.*, 1988), and as exercise continues beyond 40 minutes, uptake of gluconeogenic precursors such as lactate, pyruvate and alanine continues to increase (Ahlborg and Felig, 1982; Wahren *et al.*, 1971). By the end of prolonged exercise, production of glucose via gluconeogenesis may represent 20 to 50% of total hepatic glucose released (Ahlborg and Felig, 1982). There is a maximal rate of glucose production via gluconeogenesis during exercise and as a result, if exercise continues for a long time (~3 h) and hepatic glycogen stores are near depletion, R_a glucose will decrease and hypoglycemia can develop (Ahlborg and Felig, 1982).

Mammalian studies indicate that R_a glucose is stimulated during exercise due to increasing neuronal output and circulating hormones such as the catecholamines and glucagon (Hoelzer *et al.*, 1986; Issekutz and Vranic, 1980; Moates *et al.*, 1988; Wasserman *et al.*, 1989). Moates *et al.* (1988) used adrenalectomised dogs to show that for at least the first 90 minutes of exercise glucagon is more important, yet later on an increase in plasma EPI is required to increase lactate availability as a gluconeogenic precursor. In contrast to dogs, studies on exercising humans show that catecholamines

are of primary importance for initiating increased glucose production (Coggan, 1991). Although it is often assumed that levels of circulating catecholamines rise during sustained exercise in fish, this has not been clearly demonstrated. Some studies show an increase in plasma catecholamines but these reports involve grasping the tail of the fish, a stress that itself quickly increases the plasma EPI level (Nakano and Tomplinson, 1967; Opdyke *et al.*, 1982). However, if plasma samples are taken from cannulated trout, they actually show a decrease change in circulating catecholamines during sustained exercise (Butler *et al.*, 1986).

3.2 Epinephrine

Very few studies have looked at the effects of EPI on glucose metabolism *in vivo* in fish (Steffensen *et al.*, 1987; Van Raaij *et al.*, 1995; Wright *et al.*, 1989). These studies have shown that EPI causes hyperglycemia and stimulates glycogenolysis and gluconeogenesis in the liver. However, increased activity of glycogenolysis is never accompanied by a significant depletion of hepatic glycogen. Additionally, glucose concentration and flux do not always change in parallel (Haman *et al.*, 1997b). Therefore, one cannot extrapolate that EPI will increase R_a glucose, it needs to be directly quantified *in vivo* by experimentally increasing circulating EPI concentrations while measuring R_a glucose.

Potent effects of catecholamines on intermediary metabolism by direct and indirect actions have been long appreciated in mammalian studies. The direct action of EPI involves stimulation of hepatic glucose production by stimulating of both glycogenolysis and gluconeogenesis and depression of glucose utilization by peripheral

tissues (Cherrington *et al.*, 1984; Sacca *et al.*, 1983). The increase in glycogenolysis is transient whereas gluconeogenesis is more sustained (Pagliassotti *et al.*, 1994). The indirect actions of EPI are inhibition of insulin secretion and stimulation of glucagon secretion. Insulin does increase during EPI infusion probably in response to hyperglycemia, but there is a sharp increase in plasma insulin after discontinuation of EPI infusion (Rizza *et al.*, 1980). EPI-stimulated glucagon secretion has only been found in some species (dogs and humans) since hyperglycemia is expected to suppress glucagon secretion. However, glucagon secretion is of value in a regulatory sense since it can markedly increase the gluconeogenic potential of the liver (Pagliassotti *et al.*, 1994).

The mechanisms by which EPI affects glucose metabolism is well characterized using isolated hepatocytes in fish. Once released into the circulation, EPI binds to adrenergic receptors (adrenoreceptors, AR) presumably to produce a variety of metabolic responses such as lipid mobilization and glucose mobilization. Adrenoceptors are classified as α_1 (A, B, and C), α_2 (A, B, C and D), β_1, β_2, and very recently β_3 and β_4 based upon the potency of specific adrenergic ligands and evidence from molecular biology studies (Fabbri and Moon, 1994, Duman, 1995 #1852). β- and α_2-AR have higher affinity for EPI whereas α_1-AR have a higher affinity for NEPI (Exton, 1985). Recent work has demonstrated that both α- and β-AR are present on hepatic membranes of at least the American eel, rainbow trout and bullhead (Fabbri *et al.*, 1998). It has not yet been determined if α-AR are involved in the regulation of glucose production in these species because the α-antagonist phentolamine does not block the glucose release caused by catecholamines in trout (Exton, 1985; Perry *et al.*, 1988) whereas propranolol (PROP), a β-AR antagonist, blocks this effect (Fabbri *et al.*, 1998). Therefore, it seems likely that

effects of EPI are probably mediated through β-AR.

Just recently α_1-AR were characterized in the liver of lower vertebrates (Fabbri *et al.*, 1998). Stimulation of α_1-AR causes the contraction of vascular smooth muscle, but in the liver the main response is increased glycogenolysis (Exton, 1985). The activation of α_1-AR by EPI increases hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2), through an intermediary G_q protein. This hormone-sensitive enzyme catalyzes the formation of two potent second messengers: diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). DAG remains within the membrane whereas the water soluble product IP_3 diffuses from the plasma membrane to the endoplasmic reticulum (Hadley, 1992). These two second messengers activate protein kinase C which binds to a specific IP_3 receptor and causes Ca^{2+} channels to open and sequester Ca^{2+} into the cytosol (Fabbri and Moon, 1994; Summers and McMartin, 1993). Ca^{2+} activates a variety of Ca^{2+}-dependent enzymes including the Ca^{2+}/calmodulin-dependent protein kinase that increases glycogenolytic rate by phosphorylating Phosphorylase B to Phosphorylase A (Lehninger *et al.*, 1993).

The β-AR are distributed throughout all the vertebrate classes (Fabbri and Moon, 1994) and hepatocytes of rainbow trout primarily consist of the β_2 subtype (Reid *et al.*, 1992). The action of EPI via β-AR begins with the binding of the hormone to a protein receptor in the plasma membrane of hormone-sensitive cells such as adipocytes, hepatocytes or myocytes. This noncovalent binding apparently promotes a conformational change in the intracellular domain of the receptor and allows replacement of the GDP bound to G_S by GTP, thereby activating G_S. G_S moves to a nearby integral protein of the plasma membrane, adenylate cyclase (ACase), that in turn catalyzes the

production of cAMP from ATP, raising the cytosolic level of this second messenger. Cyclic AMP is short-lived and very quickly degrades to the inactive form 5'-AMP by cyclic nucleotide phosphodiesterase. This process activates cAMP dependent protein kinase A that phosphorylates glycogen phosphorylase B, converting it into phosphorylase A, activating glycogenolysis and inactivating glycogen synthesis. The α_2-AR have similar signal transduction pathway as all β-AR (Fabbri *et al.*, 1998) except the pathway involves G_i protein instead of G_S protein. However, activation of α_2-AR results in the inhibition of ACase, that results in glycogenesis instead of glycogenolysis.

EPI also stimulates gluconeogenesis by initiating the same transduction cascades, as described previously, that lead to the inhibition of pyruvate kinase (PK) (Wright *et al.*, 1989). PK is a regulatory enzyme in both gluconeogenesis and glycolysis. In the unphosphorylated form, PK catalyzes the last step in glycolysis where the transfer of the phosphate group occurs from phosphoenolpyruvate (PEP) to ADP. cAMP-dependent protein kinase A, produced in the EPI initiated signal transduction pathway, also phosphorylates PK along with phosphorylase B. Inactivating PK blocks the conversion of PEP to pyruvate and prevents oxidation of pyruvate via the citric acid cycle. This results in the accumulation of PEP favouring gluconeogenesis (Lehninger *et al.*, 1993).

4. Hypotheses and goals of investigation

To date, hepatic glucose production has never been measured in fish during swimming. Therefore, the aim of my thesis is to quantify R_a glucose in rainbow trout swimming at submaximal velocities. I hypothesize that when fish are subjected to submaximal exercise, hepatic glucose production will rise to meet the increased energy demand of the working muscles (Chapter 2). This hypothesis is tested by measuring *in vivo* turnover rates of glucose by continuous infusion of 6-^{3}H-glucose (Haman and Weber, 1996) in fish swimming for 3 hours at 1.5 bl s^{-1} (body length per second). Changes in circulating catecholamines are also measured to determine their possible role in regulating R_a glucose during exercise.

Surprisingly, R_a glucose and levels of circulating EPI decreased during exercise (see Chapter 2). Since EPI can be a possible candidate for regulating R_a glucose in exercise, I investigated the effects of circulating EPI on R_a glucose *in vivo*: 1) by infusing EPI to increase plasma levels and 2) by blocking the β-AR mediated effects of basal EPI with PROP (Chapter 3). *In vitro* studies in which isolated hepatocytes or liver slices were exposed to high concentrations of EPI show an increase in glucose release whereas PROP (in the presence of EPI) decreases glucose release (Mommsen *et al.*, 1994; Mommsen *et al.*, 1988; Perry *et al.*, 1988). Based on these previous results, I hypothesize that: a) EPI will increase hepatic glucose production of rainbow trout *in vivo*, and b) PROP will decrease hepatic glucose production of trout *in vivo* by eliminating the effect of basal EPI levels.

Additionally, *in vivo* effects of EPI are compared with *in vitro* effects using the

same batch of fish. These two approaches are very different, but complement each other in understanding the mechanism by which EPI affects glucose metabolism. EPI and PROP are known to affect the cardiovascular and respiratory systems (Farrell and Graham, 1986; Wood, 1975; Wood and Shelton, 1980), therefore, *in vitro* experiments can help to determine the degree to which *in vitro* effects are representative of effects taking place in the intact animal. This comparison should enable us to start separating the direct and indirect effects of EPI and PROP. Here, I hypothesize that *in vivo* and *in vitro* rates of hepatic glucose production will be similar. I also hypothesize that the magnitude of the effects of EPI and PROP will not be similar *in vivo* and *in vitro* because some indirect effects of EPI (e.g. on cardiovascular and respiratory systems) will only be present *in vivo*.

In chapter 4, I discuss the regulation of hepatic glucose production by EPI and suggest possibilities for future experiments.

CHAPTER 2

EFFECTS OF SUSTAINED SWIMMING ON HEPATIC GLUCOSE PRODUCTION OF RAINBOW TROUT

Introduction

Mammals tightly control circulating glucose concentration by continuously matching rates of hepatic glucose production and peripheral utilization (Pagliassotti *et al.*, 1994). Such a regulatory process plays a fundamental role because this metabolic fuel represents an essential source of energy for the nervous system as well as for other tissues. During submaximal exercise, hepatic glucose production (R_a glucose) is usually strongly stimulated to replace the additional glucose consumed by locomotory muscles. The nature of this response has been well characterized in mammals (Wasserman and Cherrington, 1991), but little information is available for teleosts, a group of vertebrates known to tolerate wide fluctuations in glycemia (Driedzic and Hochachka, 1978; Palmer and Ryman, 1972). Baseline glucose kinetics have been quantified in a variety of fish species including American eel, sea bass, and rainbow trout (see Weber and Zwingelstein (1995) for a review), and the effects of rapid changes in environmental conditions on the R_a glucose of trout have also been investigated recently. Acute hypoxia causes a brief stimulation of hepatic glucose production leading to hyperglycemia, whereas a rapid decrease in water temperature triggers a strong decline in R_a glucose, but without affecting concentration (Haman *et al.*, 1997b). To date, all the measurements of R_a glucose in fish have been carried out in the resting state and the effects of swimming on glucose fluxes are unknown. Maximal exercise and anaerobic metabolism have been the focus of most studies of carbohydrate utilization in teleosts (Moyes and West, 1995) even though wild salmonids spend most of their time swimming at submaximal velocities (Beamish, 1978; Quinn, 1988; Weihs, 1973). The limited data presently available on

aerobic swimming suggest that lipids are oxidized preferentially, whereas the contribution of carbohydrates remains small (Driedzic and Hochachka, 1978; Moyes and West, 1995; Walton and Cowey, 1982). In contrast, the effects of sustained exercise on the R_a glucose of mammals have been thoroughly investigated. Rats (Brooks and Donovan, 1983), dogs (Weber *et al.*, 1996), and humans (Weber *et al.*, 1990) increase glucose production by 2 to 4-fold during exercise to help meeting the higher energy demands of working muscles. By analogy, I have hypothesized that hepatic glucose release of fish would also be strongly increased during sustained swimming. Therefore, our goal was to quantify R_a glucose by continuous tracer infusion before, during, and after submaximal exercise in rainbow trout. Circulating catecholamines were also measured to determine whether they could play a role in the regulation of hepatic glucose release *in vivo*. Finally, the effect of exercise on glycogen reserves was evaluated in an attempt to partition the relative contributions of glycogenolysis and gluconeogenesis to total glucose production.

Materials and Methods

Animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum) of both sexes (494-1209 g; and length: 39.1 to 42.5 cm) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in a 1300 l flow-through tank at 13^0 C. They were kept in dechloraminated, well oxygenated water under 12h L:12h D photoperiod. The animals were fed low fat Purina trout chow (composition - 40% protein, 10% fat, ~20% carbohydrate) three times a week until satiation. Fish were acclimated to these conditions for at least 6 weeks prior to experiments.

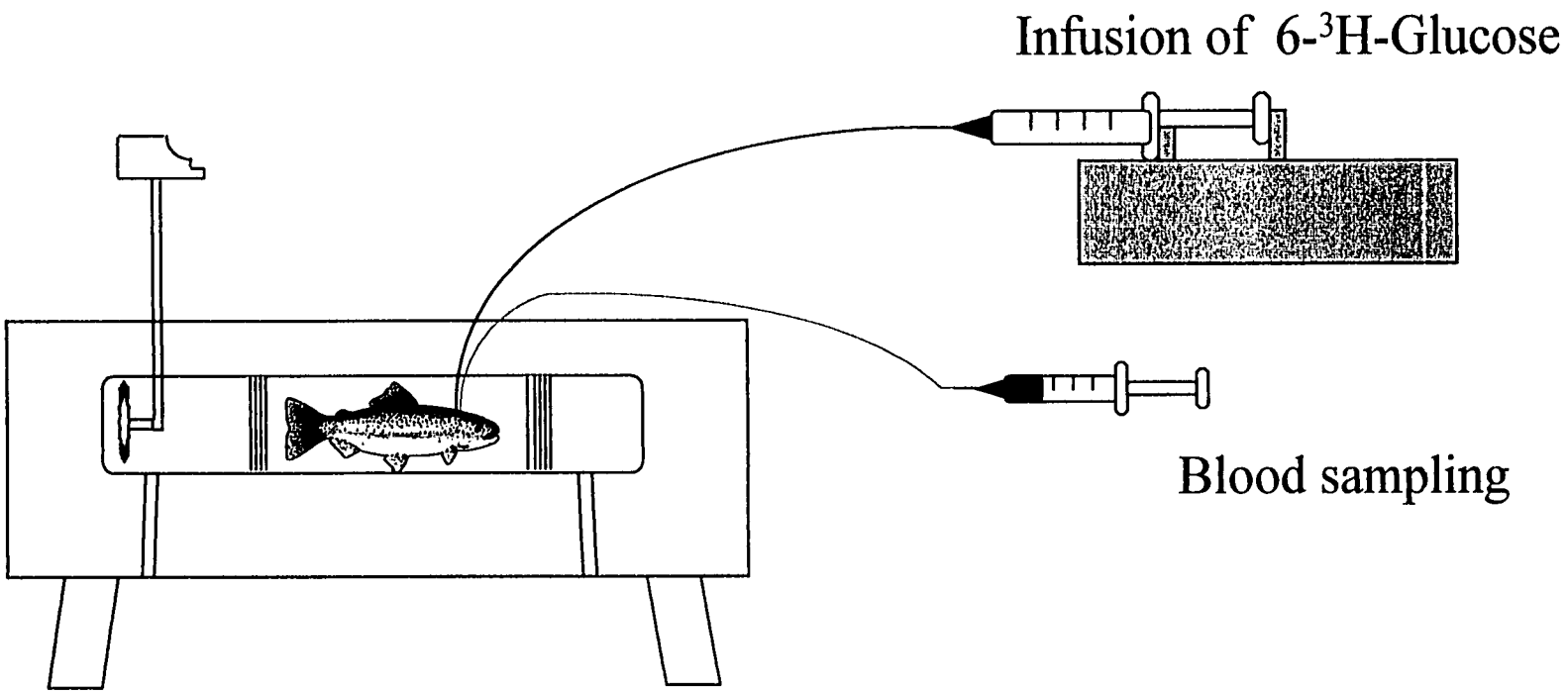
Catheterization

A double cannulation of the dorsal aorta was performed under anesthesia (0.1 g l^{-1} ethyl-N-aminobenzoate sulphonic acid, MS-222, buffered with 0.2 g l^{-1} sodium bicarbonate) (Haman and Weber, 1996). Cannulated animals were allowed to recover for at least 36 h in a swim tunnel with a weak water current (11 cm s^{-1}) to ensure adequate oxygenation. At this water flow rate, the animals were not swimming but resting on the floor of the swim tunnel.

Swim tunnel

Measurements were carried out in a modified Blazka-type swim tunnel (Beamish, 1978). It consisted of a polyvinylchloride tube (1.5 m in length and 0.2 m in

Figure 2.1 Diagram showing the swim tunnel and experimental set up. The black line represents the infusion cannula and the gray line represents the blood sampling catheter.


Infusion of 6-^{3}H-Glucose
Blood sampling

diameter) immersed in a 550-l flow-through tank. Two 'honey comb' grids were placed inside the tube to delimit a 28-l swimming chamber (0.9 m long) and to ensure laminar flow. The upper part of this chamber was closed by a transparent lid with a longitudinal slit allowing access to the catheters. Water flow was powered by an electrical trolling motor (Mini Kota, 17 lbs thrust) connected to an adjustable power supply (Harrison 6433B DC, Hewlett Packard) to control water velocity. This swim tunnel was adequate to exercise fish up to 80 cm s^{-1}. Velocity was calibrated by videotaping the repeated release of a dye suspension through the chamber and by counting film frames. For each experiment, water velocity was corrected according to Webb (1974) and Nelson *et al.* (1996), to take into account the acceleration of water around individual animals of different sizes (Bernard *et al.*, 1999). The upstream end of the swimming chamber was kept darker than the downstream section and the animals would typically swim in the upstream portion to avoid bright light.

Continuous isotope infusion

The rate of appearance of glucose (R_a glucose) was measured by continuous infusion of 6-^{3}H glucose as described (Haman and Weber, 1996) and validated (Haman *et al.*, 1997a) previously. Briefly, the infusate was prepared daily by drying the isotope supplied commercially in ethanol under N_2 at 35^{0}C, and resuspended in Cortland saline (Haman *et al.*, 1997a). While the fish was at rest, a priming dose equivalent to 90 min of infusion was injected and a continuous infusion was started at 1 ml h^{-1} using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA). Infusion rates averaged

74,286 ± 8,800 DPM kg^{-1} min^{-1}. The rate of appearance of glucose was quantified before, during, and after 3 h of swimming at 1.5 bl s^{-1} (N=7). Isotope infusion was started 1 h before the beginning of exercise and it was continued for 1 h after the end of exercise to monitor recovery. For every fish, a total of 19 blood samples of approximately 0.2 ml each were drawn throughout the infusion.

Blood sample analysis

Immediately after sampling, haematocrit was measured and the blood was centrifuged to separate plasma. The plasma was stored at -20^0 C for a maximum of 2 days before measuring lactate and glucose concentrations (Beckman DU 640 spectrophotometer at 340 nm) as well as glucose specific activity (Packard Tri-Carb 2500 scintillation counter). Glucose activity was measured by drying 30 µl of plasma under N_2 at 35^{0}C, resuspended in 1 ml water, and counting in ACS II scintillation fluid (Amersham, Oakville, Ontario, Canada). Rates of appearance (R_a) and disappearance (R_d) were first calculated with the non-steady state equation of Steele (Steele, 1959). Because R_a and R_d were never different from each other throughout the experiments ($P>0.05$), only R_a values calculated with the steady state equation are reported in this paper.

Preparation of tissue samples

The effects of exercise on glycogen stores were assessed on 16 fish from the same batch as the animals used for infusions. These individuals were also doubly cannulated and allowed to recover in the swim tunnel as described previously, but no isotope

infusion was performed. They were randomly assigned to a control and an exercise group, and were sacrificed before or after 3h of swimming at 1.5 bl s^{-1} by injecting sodium pentobarbital (65 mg/ml; MTC Pharmaceuticals, Cambridge, Ontario) into one of the catheters. Liver, white muscle, red muscle and kidney samples were excised, weighed and freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen within 4 min after death. White muscle was sampled from the anterior, middle, and posterior regions in random order (directly above the operculum, posterior to the dorsal fin, and directly below the adipose fin, respectively) to determine whether glycogen is stored uniformly along the fish. The tissues were stored at -80^0 C until further analysis. The frozen tissue samples (0.5-1.5g) were crushed to a fine powder using a mortar and pestle. And poured into a 15 mL Corex centrifuge tube. The remaining liquid nitrogen in the tube was allowed to evaporate and 4 ml of cold 8% perchloric acid (PCA) was immediately added to the tube to deproteinize the tissue. The samples were homogenized with a Polytron tissue homogenizer 30 seconds at medium speed for three times with 10 seconds of cooling down between the two-homogenization periods. An additional 1 ml of PCA was added to deproteinize the sample and rinse the sides of the tube. Two 200 µl aliquots of homogenate were removed from the Corex tubes. The remaining homogenate was centrifuged (Beckman GS-6 centrifuge) for 10 min at 3000 rpm. The supernatant, used to measure free glucose, was transferred into Eppendorf tubes and all samples were frozen until analysis could be performed. Glycogen content was determined by hydrolyzing the homogenate with amyloglucosidase (Sigma) and measuring glucose concentrations on a Packard Spectra Count Spectraphotometer at 340 nm (Bergmeyer, 1985).

During these separate experiments, blood samples were taken at t = 0, 15, 90, and

180 min of exercise. Plasma samples after centrifugation were frozen in liquid N_2 immediately and stored at -80^{0}c until further analysis. Catecholamines levels were determined on alumina extracted samples using high-pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). Internal standard used was 3,4-Dihydroxybensalamine hydrobromide.

Calculations

Glucose appearance (R_a) and disappearance (R_d) rates were calculated separately using the following non-steady state equations (Wolfe, 1992)

$$Ra = \frac{F - pV[(c_1 + c_2)/2][(SA_2 - SA_1)/(t_2 - t_1)]}{(SA_1 + SA_2)/2}$$

$$Rd = Ra - pV[(c_2 - c_1)/(t_2 - t_1)]$$

Where pV is the pool volume of the metabolite (50 ml/kg), F is the infusion rate of the isotope, SA is the metabolite plasma specific activity, c is the metabolite plasma concentration, and t is the sampling time at times *1* and *2*. Rates of appearance and disappearance were not significantly different from one another so the R_a was plotted using Steele's steady state equation (Wolfe, 1992)

$$\mathrm{Ra} = \frac{\mathrm{F}}{\mathrm{SA}}$$

Glycogen concentration was converted to glycogen reserves of each tissue with respect to the weight of the whole fish to compare total glycogen content of the different tissues.

$$\text{Glycogen reserves} = \frac{\text{glycogen concentration} \bullet \text{total mass of tissue}}{\text{mass of the whole fish}}$$

Statistical analyses

All results are presented as means ± SEM. Changes in glucose and lactate concentrations, glucose specific activity, R_a glucose, and haematocrit were assessed by Two Way Analysis of Variance (ANOVA) and Dunnett's multiple comparison tests after log 10 transformation when normality tests failed. Minimum detectable difference was calculated to determine what difference was required between the two means (Pre-exercise and exercise groups) to be significant with a given sample size. This test is done at the power of $1 - \beta = 0.90$ and at the level of $\alpha = 0.05$ significance (Zar, 1996).

Results

Plasma lactate and haematocrit

Figure 2.2 shows mean plasma lactate concentration and haematocrit of rainbow trout throughout the experiments. Lactate concentration averaged 1.11±0.03 mmol l^{-1} over time (N=19). Mean plasma lactate went from 0.97 to 1.26 mmol l^{-1} during exercise, but this small increase was not significant (P=0.13). Haematocrit showed no change during the experiments (P=0.21) and averaged 26.1±0.3% over time (N=19).

Effects of exercise on glucose kinetics

Glucose concentration, specific activity, and rate of appearance throughout the experiments are plotted in Fig. 2.3. Plasma glucose concentration averaged 3.78±0.47 mmol l^{-1} (N=19) over time and did not change during or after exercise (P=0.84). Glucose specific activity increased above resting levels at the end of exercise and during recovery (P<0.002). The rate of appearance of glucose averaged 7.6±2.1 μmol kg^{-1} min^{-1} (N=7) in the resting state and decreased during the experiments (P<0.05). Exercise caused a progressive decline in R_a glucose that became significantly lower than resting values during the last 30 min of swimming. R_a glucose remained below resting levels throughout recovery when it averaged 5.1±1.3 μmol kg^{-1} min^{-1}.

Tissue glycogen reserves

Glycogen concentration in the liver, kidney, white muscle and red muscle of non-exercised and exercised fish are presented in Table 2.1. The exercise protocol had no

Figure 2.2 Plasma lactate concentration (closed circles) and haematocrit (open circles) in rainbow trout during rest, swimming for 3 h at 1.5 BL s^{-1} and recovery. Values are means and SEM (N=7).

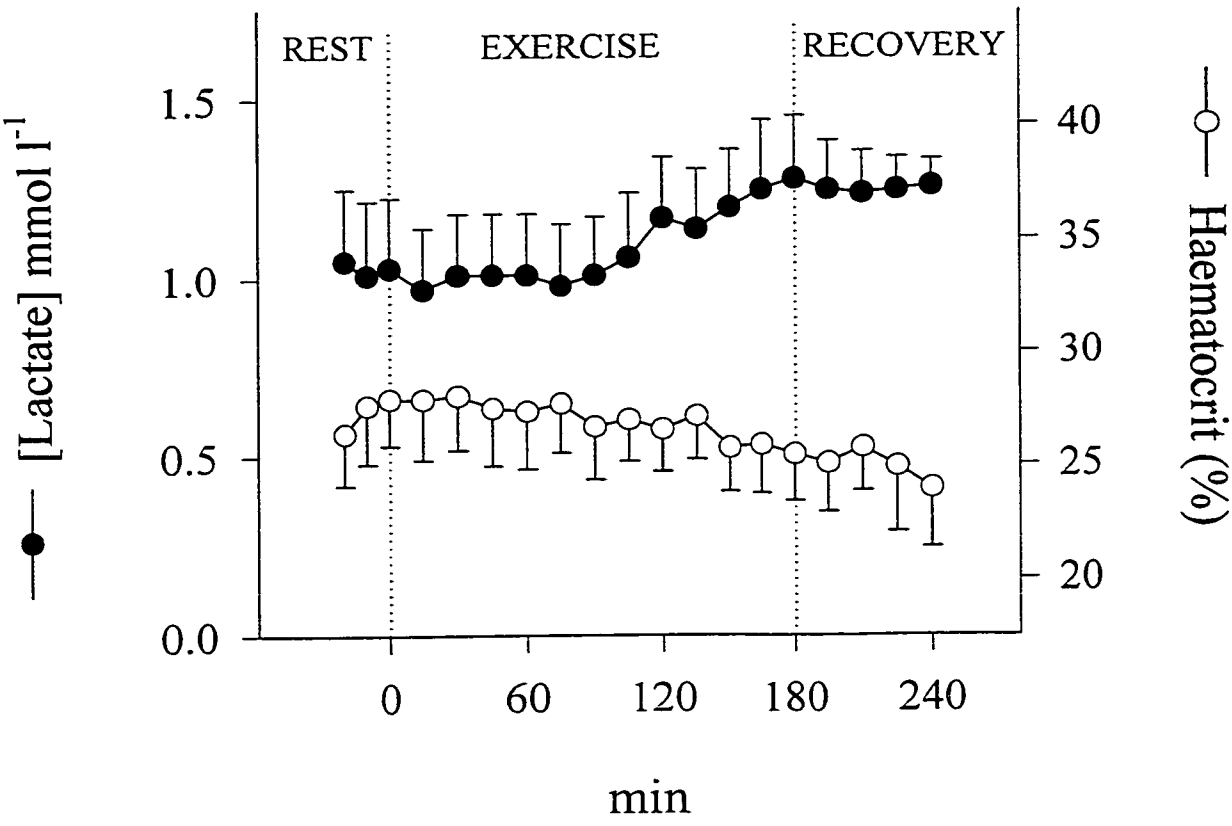

REST
EXERCISE
RECOVERY
[Lactate] mmol l-1
Haematocrit (%)
1.5
1.0
0.5
0.0
40
35
30
25
20
0
60
120
180
240
min

Figure 2.3 Plasma glucose concentration (A), glucose specific activity (B) and R_a glucose (C) in rainbow trout during rest, exercise at 1.5 BL s^{-1} and recovery. Values are means ± SEM (N=7). * denote significant differences from the mean resting value ($P<0.05$).

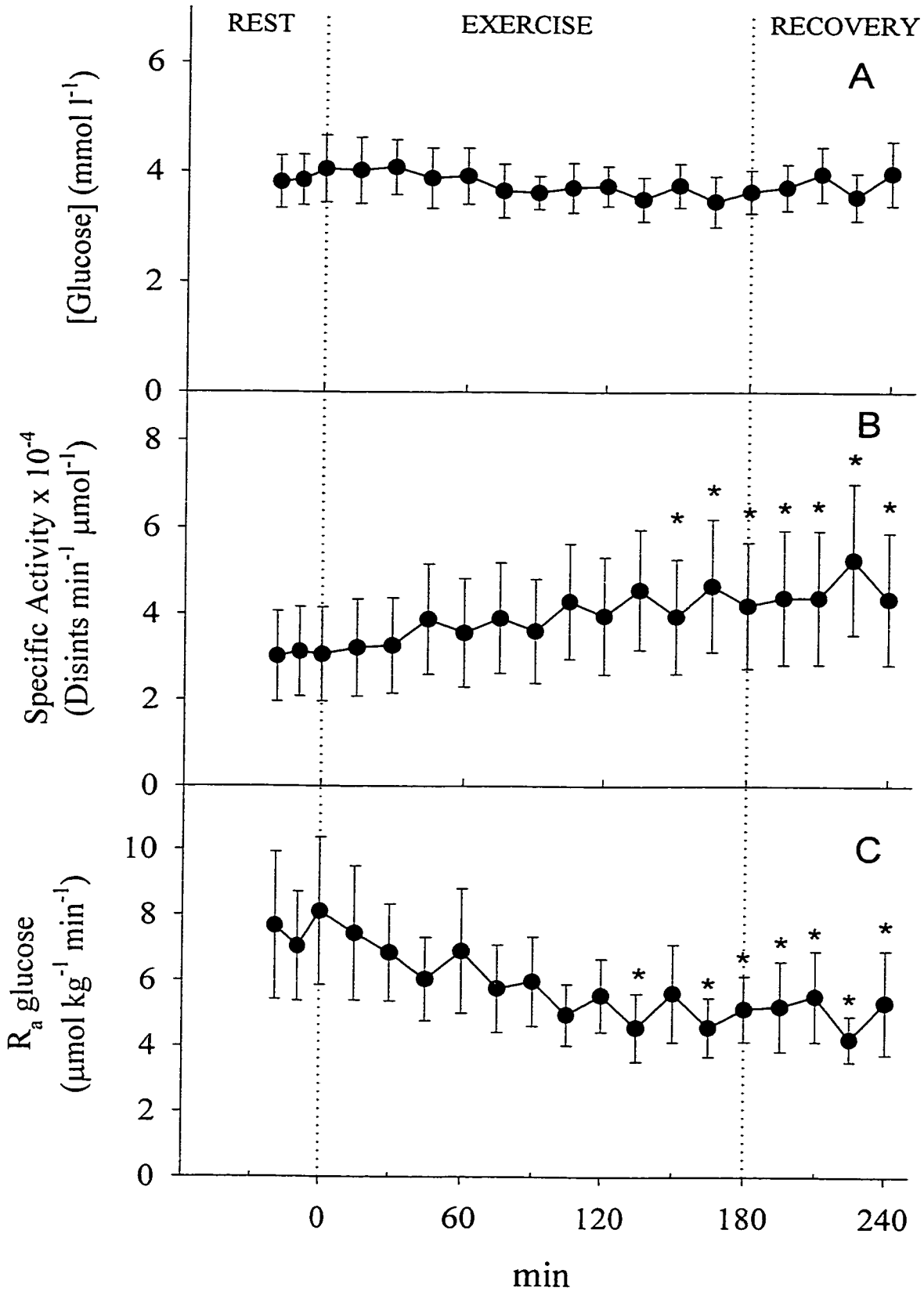

REST
EXERCISE
RECOVERY
A
B
C
[Glucose] (mmol l-1)
Specific Activity x 10-4
(Disints min-1 µmol-1)
Ra glucose
(µmol kg-1 min-1)
0
2
4
6
8
10
0
60
120
180
240
min

Figure 2.4 Plasma concentrations of epinephrine (closed circles) and norepinephrine (open circles) in rainbow trout before (time 0) and during swimming at 1.5 BL s^{-1}. Values are means ± SEM (N=8). * denote values significantly different from rest ($P<0.05$).

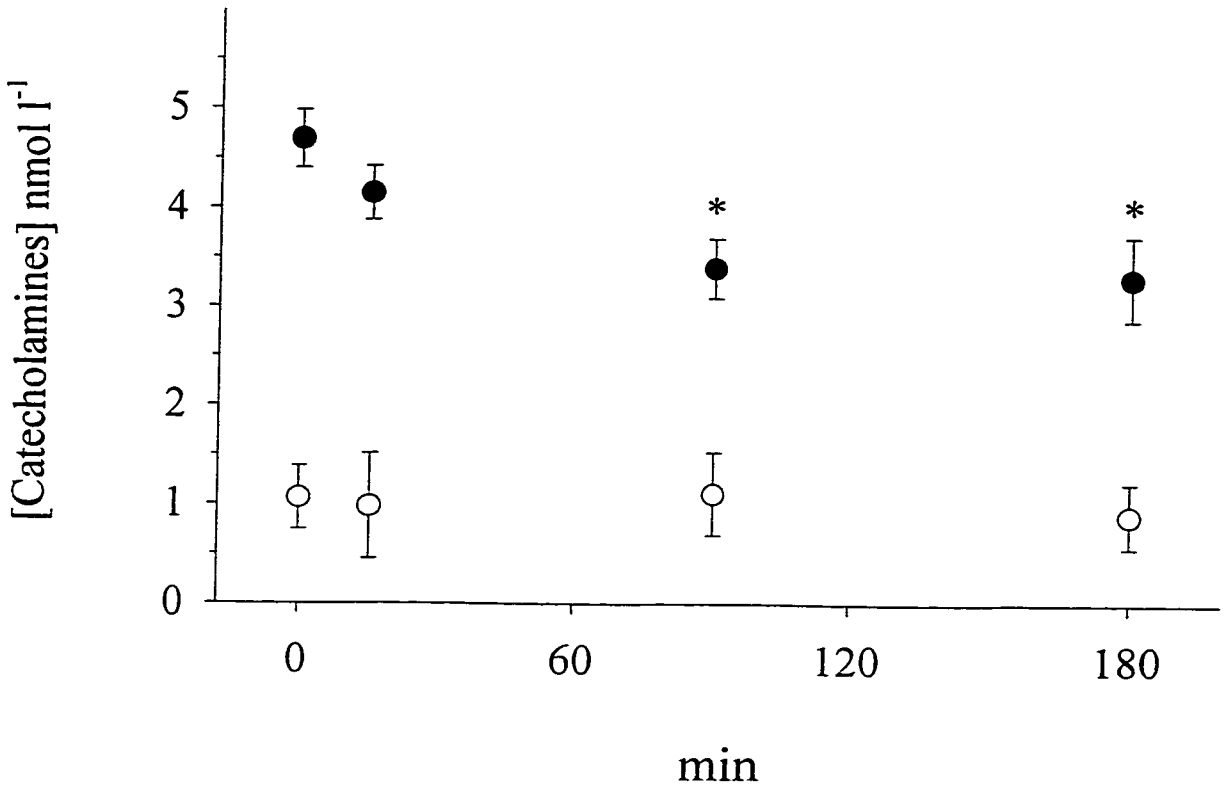

[Catecholamines] nmol l-1
5
4
3
2
1
0
*
*
0
60
120
180
min

Table 2.1 A. Average mass (in grams) of the whole fish, liver and kidney in non-exercised and exercised groups. White muscle, red muscle mass was assumed to be 60% and 5% of the body mass, respectively. B. Glycogen concentration (μmol glucosyl units g^{-1} wet wt. of tissue) and glycogen reserves (μmol glucosyl units g^{-1} of fish) in the non-exercised and exercised groups. The sample size for each tissue is N=8 except for red muscle pre-exercise N= 4 and exercised group is N=6. * denote statistical significance ($P < 0.05$)

A

	Mass (g)	
	Non-exercised	Exercised
Whole body	935.88±66.39	890.25±66.24
Liver	11.49±1.00	10.74±1.56
Kidney	5.58±0.43	5.15±0.48

B

Tissue	Glycogen Concentration μmol glucosyl units g^{-1} wet wt. tissue		Glycogen Reserves μmol glucosyl units g^{-1} fish	
	Non-exercised	Exercised	Non-exercised	Exercised
Liver	118.33±4.58	107.41±1.84*	1.49±0.18	1.27±0.12
Kidney	40.45±1.68	38.22±1.29	0.25±0.03	0.22±0.02
White Muscle	59.36±2.01	54.11±3.08	29.67±0.99	27.03±1.53
Red Muscle	137.29±9.38	58.73±2.43*	6.86±0.47	2.93±0.12*

effect on the glycogen concentration of kidney ($P=0.45$) and white muscle ($P=0.16$), but caused a significant decrease in the liver ($P<0.05$) and in red muscle ($P<0.01$). Mass-specific glycogen reserves of all tissues measured were calculated by multiplying concentration by tissue mass and dividing by total body mass (Table 2.1). Exercise only caused significant glycogen depletion in the red muscle ($P<0.01$).

Plasma catecholamines

Plasma norepinephrine and EPI concentrations before and during exercise are plotted in Fig. 2.4. Swimming caused no change in norepinephrine levels ($P=0.92$) that averaged 1.0 ± 0.4 nmol l^{-1} over time. However, there was a significant decline in EPI concentration after 90 and 180 min of exercise ($P<0.001$). EPI concentration was 4.7 ± 0.3 nmol l^{-1} at rest and decreased to 3.3 ± 0.4 nmol l^{-1} after 3 h of exercise.

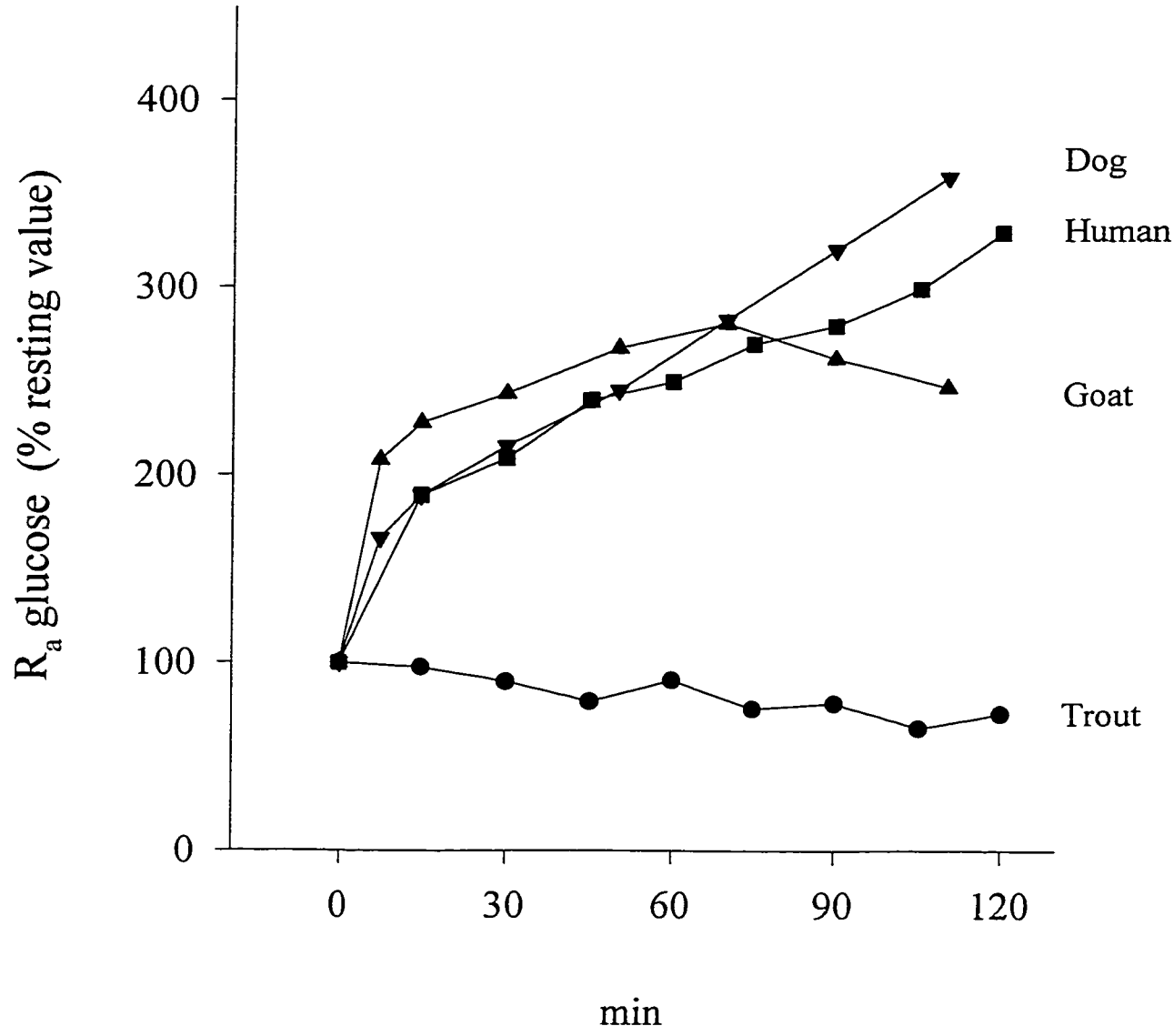
Discussion

This study is the first to provide *in vivo* measurements of the effects of exercise on the glucose kinetics of an ectotherm. Contrary to expectation, I found that sustained swimming does not stimulate hepatic glucose release in rainbow trout, but that it causes a significant decrease. Figure 2.5 summarizes the effects of submaximal exercise on the R_a glucose of several vertebrate species studied to date and it shows that the responses of mammals and rainbow trout to the same work intensity are opposite. From previous investigations of exercising salmonids of identical size, I can estimate that 1.5 BL s^{-1} corresponds to 50% $\dot{M}O_2$ max or 70% of critical swimming speed (U_{crit}) (Beamish, 1978; Kiceniuk and Jones, 1977; Webb, 1971). Submaximal exercise causes a major increase in the R_a glucose of dogs (Weber *et al.*, 1996), goats (Weber *et al.*, 1996), and humans (Coggan *et al.*, 1995), whereas a 33% decrease was observed here after 3 h of swimming (Fig. 2.3C). Furthermore, the effect of exercise was longer lasting in rainbow trout than previously observed in mammals. The low R_a glucose recorded at the end of swimming persisted throughout the 60 min recovery (Fig. 2.3C), whereas a 30 min post-exercise resting period is sufficient to return to basal levels in mammals (Weber *et al.*, 1990; Weber *et al.*, 1996).

Possible reasons for the decline in R_a glucose

It could be argued that glucose production was stimulated at the beginning of our experiments because the animals were stressed and not really at rest before swimming. However, this scenario is unrealistic because the mean R_a glucose measured in the swim tunnel before exercise (7.6 ± 2.0 μmol $kg^{-1}min^{-1}$) was similar to resting values from

Figure 2.5 Relative effect of sustained exercise (40-50% $\dot{M}_{O_2}$max) on hepatic glucose production in rainbow trout (this study) and in mammals (calculated from published values on dogs (Weber *et al.*, 1996), goats (Weber *et al.*, 1996) and humans (Coggan *et al.*, 1995)).


400
300
200
100
0
R_a glucose (% resting value)
0
30
60
90
120
min
Dog
Human
Goat
Trout

previous experiments where trout were kept quietly in opaque acrylic boxes for more than 36 h (9.0 ± 0.7 (Haman and Weber, 1996) or 8.6 ± 1.3 (Haman *et al.*, 1997b)) and where they had true resting metabolic rates (41 μmol O_2 kg^{-1} min^{-1}). Instead, changes in liver perfusion or in the concentration of circulating hormones may be responsible for the observed decrease in R_a glucose. Sustained swimming is known to cause a decline in liver blood flow by constricting the coeliacomesenteric artery (Randall and Daxboeck, 1982) and this cardiovascular response could lead to a reduction in hepatic glucose release into the circulation. A decrease in circulating catecholamines could also contribute to lowering R_a glucose during exercise. Swimming had no effect on plasma norepinephrine, but EPI levels declined by 30% (Fig. 2.4). These findings are compatible with results from another study where the plasma catecholamines of catheterized rainbow trout also decreased during prolonged exercise (Butler *et al.*, 1986). Such hormonal changes are known to reduce rates of glycogenolysis and gluconeogenesis in isolated hepatocytes (Wright *et al.*, 1989), and they may have the same effects *in vivo*.

Some can argue that these results are not valid because control experiments were not performed and that the decline in R_a glucose is due to repetitive blood sampling. I admit it would have been better to perform control experiments where R_a glucose is measured during basal state of fish to ensure that blood sampling does not affect R_a glucose. However, these experiments are expensive because of use of the radioactive isotopes. As well, due to time and money restrictions, I decided not to repeat the experiments already performed for chapter 3. Moreover, control experiments in chapter 3 (Fig. 3.2) clearly show that blood sampling does not affect circulating glucose concentration, specific activity and therefore, rate of hepatic glucose production. These

control experiments surely indicate that decline in R_a glucose is due to exercise and not blood sampling.

Glucoregulation

Frequent blood sampling allowed the close monitoring of circulating glucose concentration. Rainbow trout were able to maintain a steady glucose concentration during and after sustained exercise (Fig. 2.3A) even though glucose fluxes decreased by more than 30% (Fig. 2.3C). This homeostatic response was achieved because the progressive decrease in hepatic glucose production (R_a) was accompanied by a parallel and synchronized decline in glucose uptake by peripheral tissues (R_d). Such tight matching between the rates of appearance and disappearance of glucose is rather surprising in an animal that is often described as a poor glucoregulator (Driedzic and Hochachka, 1978; Van den Thillart, 1986). The effects of sustained swimming on the blood glucose levels of rainbow trout were only measured by a few others (Nielsen *et al.*, 1994; Zelnik and Goldspink, 1981). In these experiments, temporary hyperglycemia was observed at the beginning of exercise before blood glucose returned to baseline concentrations within a few minutes. The high plasma cortisol levels reported in these studies suggest that the transient increase in blood glucose concentration may have been caused by stress rather than exercise *per se* (Mazeaud and Mazeaud, 1981). Here, cortisol levels were not measured but I show that rainbow trout have the capacity to control blood glucose concentration during submaximal exercise when R_a glucose changes significantly over time.

Circulating glucose as an oxidative fuel to support swimming

The decrease in hepatic glucose production measured during swimming (Fig.

2.3C) shows that circulating glucose is probably not an important fuel for the locomotory muscles of rainbow trout during sustained exercise. Reducing the rate of glucose mobilization would not make much sense if glucose utilization by contracting muscles was increased during swimming. Another line of evidence supporting the same view comes from the observation that the rates of glucose disappearance (R_d glucose) and of glucose oxidation always change in parallel. In fact, R_d glucose is often considered to be a close approximation of oxidation rate during exercise because more than 90% of R_d is channeled towards oxidation in studies where oxidation and R_d have been measured simultaneously (Coggan *et al.*, 1990; Paul and Issekutz, 1967). Situations where rates of disappearance and oxidation change in opposite directions have never been observed. However, it is important to note that all the information available on this issue comes from mammalian experiments and that glucose oxidation has never been measured directly in swimming trout. Further experiments will be needed to quantify glucose oxidation in exercising fish and to confirm that it decreases below resting values as suggested here.

Mobilization of oxidative fuels other than glucose

If working muscles do not increase circulating glucose utilization during sustained exercise, other sources of energy must be mobilized. The alternative fuels available are muscle glycogen and whole-body reserves of lipids and proteins (Moyes and West, 1995; Weber and Zwingelstein, 1995). Results show that the glycogen stores of red muscle are being used significantly during swimming (Table 2.1). However, no glycogen depletion could be demonstrated in white muscle, even though this tissue is known to be recruited

at all work intensities above 35% U_{crit} (Hudson, 1973).

Recent experiments reveal that the rates of appearance of fatty acids and glycerol are not affected by endurance exercise in rainbow trout (Bernard *et al.*, 1999). Lipolysis is not stimulated beyond resting levels, even after 4 days of continuous swimming. However, a very active triacylglycerol:fatty acid substrate cycle is maintained at all times, presumably to allow rapid changes in the composition of membrane phospholipids (Bernard *et al.*, 1999). These results suggest that fatty acid oxidation does not increase during sustained swimming, but this conclusion must remain tentative because rates of fatty acid oxidation have not been measured in fish. Similarly, I can only speculate on the potential mobilization of protein reserves during sustained swimming because the fluxes of amino acid have never been quantified in an exercising teleost (Weber and Zwingelstein, 1995).

Sources of circulating glucose

All the glucose released in the circulation can be accounted for by two processes: gluconeogenesis and glycogenolysis. Therefore, the relative contribution of gluconeogenesis can be estimated by subtracting glycogenolysis from total glucose production (R_a glucose). In this context, I have tried to measure glycogenolysis by estimating glycogen depletion in liver and kidney during exercise. Unfortunately, no significant change could be demonstrated because of the high variability among individuals (Table 2.1). With the observed difference in mean glycogen depletion and the associated variances, a sample size of 118 fish would be required in each group to show a significant decline ($p<0.05$) at a power ($1-\beta$) of 0.90 (Zar, 1996). Given the observed

means and variances in pre-exercise glycogen reserves of liver and kidney, I can determine that the *minimal detectable difference* ($P<0.05$) in combined hepatic and renal glycogen reserves is 609 μmol glucosyl units per kg of fish (Zar, 1996). Any actual depletion below this value would therefore have remained undetected in our experiments. Therefore, this amount of 609 μmol glucosyl units per kg represents the maximal possible contribution of glycogenolysis to total glucose production during the 3 h of exercise. By measuring the surface area under the R_a glucose curve during exercise (Fig. 2.3C), I can also determine that total glucose production for 3 h of swimming was 1062 μmol glucosyl units per kg of fish. Therefore, the maximum possible contribution of glycogenolysis to R_a glucose was 57% (609/1062), and, by deduction, at least 43% of R_a glucose was accounted for by gluconeogenesis.

In conclusion, this study shows that prolonged submaximal exercise causes a more than 30% decline in the R_a glucose of rainbow trout and that circulating EPI may be partly responsible for controlling this response. Furthermore, trout is able to maintain steady blood glucose concentration by matching rates of glucose production (R_a) and peripheral glucose utilization (R_d) throughout exercise. The observed reduction in the R_a glucose of swimming trout is strikingly different from the 2 to 4-fold increase taking place in mammals exercising at the same intensity. These results show that circulating glucose is probably not an important oxidative fuel for sustained locomotion in fish.

CHAPTER 3

REGULATION OF GLUCOSE PRODUCTION IN RAINBOW TROUT: ROLE OF EPINEPHRINE *IN VIVO* AND IN ISOLATED HEPATOCYTES

INTRODUCTION

The hormonal regulation of fish carbohydrate metabolism has received a lot of attention over the last two decades (Mommsen and Plisetskaya, 1991; Moon and Foster, 1995; Plisetskaya and Mommsen, 1996), and numerous *in vitro* studies have investigated the specific role of catecholamines in the mobilization of glucose (Fabbri *et al.*, 1998). Epinephrine (EPI), norepinephrine, glucagon and cortisol have all been shown to increase glucose production in isolated hepatocytes from a variety of teleosts (Fabbri *et al.*, 1998; Mommsen *et al.*, 1991; Moon, 1998; Reid *et al.*, 1992). At the whole organism level, stresses such as exhaustive swimming (Milligan, 1996) and environmental hypoxia (Montpetit and Perry, 1998) stimulate EPI release, and the administration of exogenous EPI is known to cause hyperglycemia (Fabbri *et al.*, 1998). This elevation in blood glucose could be due to an increase in the rate of appearance (R_a glucose), a decrease in the rate of disappearance (R_d glucose), or a combination of both as in mammals (Cherrington, 1994). Recent experiments on rainbow trout show that prolonged exercise causes a parallel decline in R_a glucose and plasma EPI below normal resting levels (Shanghavi and Weber, 1999), suggesting that this hormone may be involved in maintaining basal R_a glucose in fish. Circulating EPI does not play such a role in mammals because the administration of α or β-adrenergic antagonists fails to reduce basal glucose production (Cherrington, 1994). The effects of EPI on fish hepatocytes are thought to be primarily mediated through β-adrenoreceptors because they are abolished in the presence of the β-blocker propranolol (PROP) (Fabbri *et al.*, 1998). The role of basal or elevated plasma EPI levels on hepatic glucose production has never been examined *in*

vivo in teleosts.

In this study, we ran parallel experiments on intact fish and on isolated hepatocytes from the same batch of animals for several reasons. Comparing values of basal R_a glucose previously measured *in vivo* (Haman and Weber, 1996; Haman *et al.*, 1997b; Shanghavi and Weber, 1999) and *in vitro* (Dugan and Moon, 1998; Mommsen, 1986; Mommsen *et al.*, 1988) reveals that rainbow trout hepatocytes seem to produce glucose more rapidly in the intact animal than after isolation. The significance of this discrepancy is not clear because the animals used for these various measurements were fed different diets, they were kept at different temperatures, and they had different body sizes (*in vivo* experiments typically requiring larger fish to perform catheterizations). Moreover, *in vivo* EPI and PROP treatments are known to influence the levels of several other hormones, and to affect the respiratory and cardiovascular systems, at least in mammals (Nijkamp *et al.*, 1992). A dual *in vivo-in vitro* approach on the same batch of animals would allow standardization of experimental conditions (diet, temperature and body size) and separation of the direct and indirect effects of EPI and PROP. Therefore, the goal of this study was to quantify the role of EPI in the regulation of hepatic glucose production in rainbow trout, and to test the following hypotheses by elevating EPI concentration and/or blocking its β-adrenergic effects in intact animals or in isolated hepatocytes: i) elevated EPI levels cause hyperglycemia by increasing R_a glucose and decreasing R_d glucose, and ii) basal plasma EPI is not involved in maintaining resting R_a glucose. We have also examined whether trout hepatocytes can produce glucose at similar rates *in vivo* and *in vitro* when diet, temperature and body size are standardized. This comparison was made to determine the integrated effect of the cell isolation

procedure (e.g. destruction of membrane integrity, abnormal stimulation of glycogen breakdown (Mommsen *et al.*, 1988), elimination of neural input) to evaluate how relevant *in vitro* measurements of glucose production are to the intact organism.

Material and Methods

Animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum) of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in a 1300 l flow-through tank at 13 °C. They were kept in dechloraminated, well oxygenated City of Ottawa water under 12h L : 12h D photoperiod. The animals were fed low fat Purina trout chow (composition: 40% protein, 10% fat, ~20% carbohydrate) or Martin trout feed (composition: 42% protein, 11% fat, ~20% carbohydrate) until satiation three times a week. Fish were acclimated to these conditions for at least 6 weeks prior to experiments and were randomly divided in 2 groups for the *in vivo* and *in vitro* experiments. *In vivo* experiments were conducted between October 1997 and September 1998. *In vitro* experiments were performed between July 1997 and August 1998.

In vivo experiments

Catheterization

The double cannulation of the dorsal aorta was performed as described in chapter 2. Cannulated animals were allowed to recover for at least 36 h in an opaque Plexiglass boxes (60 x 16 x 18 cm or 58 x 13 x 19.5 cm) with a weak water current to ensure adequate oxygenation. Fish with hematocrits lower than 20% after recovery from surgery were not used. During the exeperiments, the boxes were tightly sealed with an opaque lid to prevent exchange with atmospheric air to allow the measurement of oxygen consumption ($\dot{M}_{O_2}$) (see references (Haman *et al.*, 1997b; Steffensen, 1989) for detail).

The lid had a small opening to access the catheters.

Metabolic rate measurements

Metabolic rate was measured to observe possible respiratory effects of EPI and PROP in an intact fish. During most of the experiments, oxygen consumption ($\dot{M}_{O_2}$) was measured by interrupting the external water supply for 15 min and recycling the water within a 13.5 or 15 l closed system. Care was taken to eliminate air bubbles and to avoid exchange between the re-circulated water and atmospheric air. The water flow rate through the chamber remained at 6 to 8 l min^{-1} during metabolic rate measurements. O_2 concentration (mg l^{-1}) was recorded every minute with a calibrated oxygen electrode (Oxyguard, Handy MKIII, Valox). $\dot{M}_{O_2}$ was calculated using the closed system equation of Steffensen (1989). After each measurement, water P_{O2} was returned to normal by terminating re-circulation and flushing the chamber for at least 15 min with normoxic water. Measurements were made at t = -50.5, -20.5, 23.5, 53.5, 98.5 and 143.5 min.

Measurement of glucose kinetics

Hepatic glucose production (R_a glucose) was measured by continuous infusions of 6-^{3}H glucose as described and validated previously (Haman *et al.*, 1997a; Haman and Weber, 1996). The infusate was prepared as described in Chapter 2. Tracer infusion was started 1 h before treatment with vehicle (saline control), epinephrine (EPI) or propranolol (PROP) to quantify baseline glucose kinetics. Infusion rates averaged 95 998 ± 7 546, 88 343 ± 10 798 and 80 680 ± 7 804 DPM kg^{-1} min^{-1} for vehicle, epinephrine (EPI), and propranolol (PROP) infusion experiments, respectively. R_a presented in this

paper is representative of glucose production from only liver. The contribution from the kidney was considered to be 10.5% (Haman *et al.*, 1997a) and subtracted from the total glucose production calculated with Steele's equation to compare with rate of glucose production in isolated hepatocytes.

Infusion of vehicle, epinephrine and propranolol

One hour after starting the tracer infusion, a second syringe pump was activated to infuse vehicle (N=3, mean body mass = 566 ± 19 g), EPI (N=7, 694 ± 58 g), or PROP (N=10, 611 ± 63 g) at the rate of 1 ml h^{-1}. EPI was infused for 35 min at a rate of 1.34 nmol kg^{-1} min^{-1}. These values were selected after preliminary experiments showed that they were adequate to reach final plasma EPI concentration of approximately 500 nmol l^{-1}, thereby stimulating physiological levels normally reached after exhaustive swimming. PROP was infused at the rate of 0.113 μmol kg^{-1} min^{-1} for an hour to achieve the final concentration of 50 mmol l^{-1}. Preliminary measurements showed that this amount of PROP was sufficient to block β-AR completely because the rapid decline in arterial pH normally caused by the injection of EPI was completely abolished by this PROP treatment. Finally, tracer infusion was continued for 2 h after stopping the administration of vehicle, EPI or PROP to monitor recovery. The infusions were performed under red, low intensity light with the catheters covered by an opaque plastic sheet to avoid light-induced breakdown of EPI.

Blood sampling

In each experiment, 12-13 blood samples (0.1-0.2 ml each) were taken before,

during and after the administration of saline, EPI or PROP and they were centrifuged immediately. Plasma was stored at –80 ^{0}C before measuring glucose concentration spectrophotometrically at 340 nm (Beckman DU 640) and glucose activity by scintillation counting (Packard Tri-Carb 2500). Activity was quantified by drying 15 µl of plasma under N_2, resuspending in 0.5 ml water and counting in Safety-Solve scintillant (Research Products International, Illinois). In the EPI infusion experiments, plasma samples were measured for catecholamines concentration after 2 to 80-fold dilution. The analysis of plasma catecholamines was as described in chapter 2.

In vitro experiments

Hepatocyte isolation

The fish (N=22, mean body mass 715.5 ± 47.2 g) were killed by a blow to the head, and mid-ventral incision was made to expose the liver. Hepatocytes were isolated according to Moon *et al.* (1985). The composition of the solutions used is listed in Table 3.1.

Table 3.1: Solution composition for the isolation of hepatocytes in rainbow trout. All the concentrations are final. Solution A was made without $NaHCO_3$ and EGTA as 5X and stored at 4 ^{0}C. All the solutions had pH adjusted to 7.63 at room temperature after which they were kept on ice.

Solution	Composition
A	136.70 mmol l^{-1} NaCl 0.80 mmol l^{-1} $MgSO_4$ 0.33 mmol l^{-1} NaH_2PO_4 0.44 mmol l^{-1} KH_2PO_4 5.40 mmol l^{-1} KCl 5.00 mmol l^{-1} HEPES 5.00 mmol l^{-1} HEPES-Na 5.00 mmol l^{-1} $NaHCO_3$ 1.00 mmol l^{-1} EGTA
B	Solution A (without EGTA) 30mg/100 ml collagenase
C	Solution A (without EGTA) 2% Bovine serum albumin (BSA) 1.5 mmol l^{-1} $CaCl_2$ 2.0 mmol l^{-1} Alanine 1.5 mmol l^{-1} Lactate

The hepatic portal vein or hepatic artery was catheterized and solution A (See Table 3.1) was pumped through the liver at 7 to 8 ml min^{-1} with a Monostat Varistaltic Pump (AL Series) for 2 to 5 min to clear the liver of blood. Solution B was then pumped in for an additional 20 to 25 min to digest the liver. After the perfusion, the liver was removed and the gallbladder was left intact in the animal. The liver was first weighed in a plastic petri dish and then, finely chopped with a razor blade in solution A and filtered through two nylon screens (253 and 73 μm). Isolated hepatocytes were rinsed twice with Solution A by centrifuging at 90 g or 1000 rpm at approximately 13 ^{0}C for 2 min in a Sorvall RC5B plus centrifuge (SS34 rotor; Du Pont Canada). The third rinsing of hepatocytes was performed with half volume of solution A and half volume of solution C. The fourth rinse was only with solution C. All centrifugations were for 2 min and at 1000 rpm. The final pellet was resuspended in the solution C and left on ice for approximately 1 h. After 1 h of 'metabolic rest', hepatocytes were centrifuged again for 1 min. The pellet was resuspended in a small amount of solution C. The cells were counted with a haemocytometer and viability was measured via the trypan blue exclusion method. The hepatocytes were only used if the viability was greater than 80%.

Hepatocytes incubation

For each treatment, 50 mg of cells were incubated for 15-60 min at 13 ^{0}C with solution C alone (control) or added with EPI, PROP or both. The pH of the solution was adjusted to 7.63. Incubations with EPI (bi-tartrate salt, Sigma) were performed at low (5 nmol l^{-1}) or high concentrations (500 nmol l^{-1}) to stimulate normal levels at rest (low EPI) or after exhaustive exercise (high EPI). Incubations with PROP (bi-tartrate salt, Sigma)

were performed at final concentration of 50 mmol l^{-1}. In experiments where both PROP and EPI were used, PROP was added 15 min before EPI to ensure that all β-AR were blocked. Incubations were carried out in a 24 well disposable plastic dish covered with aluminium foil to avoid light exposure (especially for EPI). The dishes were slowly shaken in a water bath shaker at 13 ^{0}C to ensure that the cells would not settle to the bottom. Incubations for each treatment were terminated with the addition of perchloric acid (final concentration = 6%) every 15 min until 1 h. Samples were then centrifuged for 6 min at room temperature in an Eppendorf centrifuge (Eppendorf Centrifuge Model 545C). The resulting supernatants were frozen for later analysis of glucose concentrations and total glucose release was calculated after. The glucose concentration was measured spectrophotometrically on a Beckman DU 640 at 340 nm (Bergmeyer, 1985).

These experiments were divided into two groups. The first set consisted of control, PROP, and high EPI treatments (N=11, mean body mass 807 ± 70 g). The second set consisted of control, low EPI, high EPI, low EPI + PROP and high EPI + PROP (N=11, mean body mass 624 ± 54 g).

Calculations and statistics

All *in vivo* R_a glucose values reported in this paper were calculated with the steady state equation of Steel (1959) because they were never significantly different from R_d glucose when both rates were calculated separately with the non-steady state equation. To allow meaningful comparisons with *in vivo* R_a glucose, *in vitro* rates of glucose production measured in umol glucose produced per g hepatocytes were converted to μmol

kg^{-1} min^{-1} as follows:

$$\textit{In vitro}\ R_a\ \text{glucose} = \frac{\text{Glucose produced (umol } g^{-1} \text{ hepatocytes) x 0.90 liver mass (g)}}{\text{Body mass (g) x incubation time (min)}}$$

Liver mass was multiplied by 0.9 because only ~90% of all liver cells are hepatocytes that can contribute to glucose production. For all the comparisons between rates of glucose production measured *in vivo* and *in vitro*, measured *in vivo* R_a values were corrected for renal glucose production by subtracting 10.5% (Haman *et al.*, 1997a).

For the *in vivo* experiments, changes in $\dot{M}_{O_2}$, glucose concentrations, glucose specific activity and R_a glucose were assessed by two-way ANOVA and Dunnett's multiple comparison or Tukey's tests. Differences between *in vitro* treatment and comparisons between R_a values measured *in vivo* and *in vitro* were tested with one-way ANOVA or Kruskal-Wallis one way ANOVA on ranks when normality tests failed. Percentages were converted to the arcsine of their square root before analysis. All statistical tests were performed using computer software Sigma Stat 2.0 (Jandel Scientific). All the values presented are means ± SEM.

RESULTS

In vivo experiments

Control saline inufsions

Infusion of vehicle saline had no effect on $\dot{M}_{O_2}$ (Fig. 3.1), plasma glucose concentration (Fig. 3.2A), glucose specific activity (Fig. 3.2B), or R_a glucose (Fig. 3.2C) ($P>0.05$). Throughout the saline infusion experiments, $\dot{M}_{O_2}$ averaged 38.69 ± 0.36 μmol O_2 kg^{-1} min^{-1} (N=6), plasma glucose concentration 5.44 ± 0.06 mM (N=13) and R_a glucose 7.95 ± 0.08 μmol kg^{-1} min^{-1} (N=13). These values were not different from mean baseline levels measured before starting the administration of EPI or PROP (Fig. 3.1, 3.4 and 3.5) ($P>0.05$). Mean hematocrit did not change significantly during the saline infusion experiments and averaged 24.9 ± 1.6 % (N=3).

Epinephrine infusion

The infusion of EPI caused an increase in $\dot{M}_{O_2}$ from a mean baseline value of 40.67 ± 0.39 μmol O_2 kg^{-1} min^{-1} to 61.36 ± 2.67 (N=7, $P<0.05$), and $\dot{M}_{O_2}$ was still significantly higher than baseline 1 h after the end of EPI infusion ($P<0.05$, Fig. 3.1). Figure 3.3 shows plasma catecholamine concentrations before, during, and after EPI infusion. Plasma EPI was 2.36 ± 0.72 nM (N=7) before starting the exogenous administration of EPI and increased to 545 ± 23.91 nM (N=6; $P<0.001$) after 30 min of exogenous EPI infusion. Plasma EPI concentration returned to baseline 25 min after the end of infusion and remained at basal levels until the end of the measurements. Plasma norepinephrine concentration did not change significantly throughout the experiments ($P>0.05$) and averaged 19.47 ± 5.34 nM (N=8). The effects of EPI infusion on glucose

Figure 3.1 Rates of oxygen consumption ($\dot{M}O_2$) before, during and after infusion of vehicle saline (A), epinephrine (B), or propranolol (C). Shaded areas indicate actual infusion times. Values are means ± SEM. Significant differences ($P<0.05$) are indicated as follows: a, different from mean $\dot{M}O_2$ before infusion; b, different from mean $\dot{M}O_2$ during epinephrine infusion; c, different from mean $\dot{M}O_2$ during propranolol infusion.

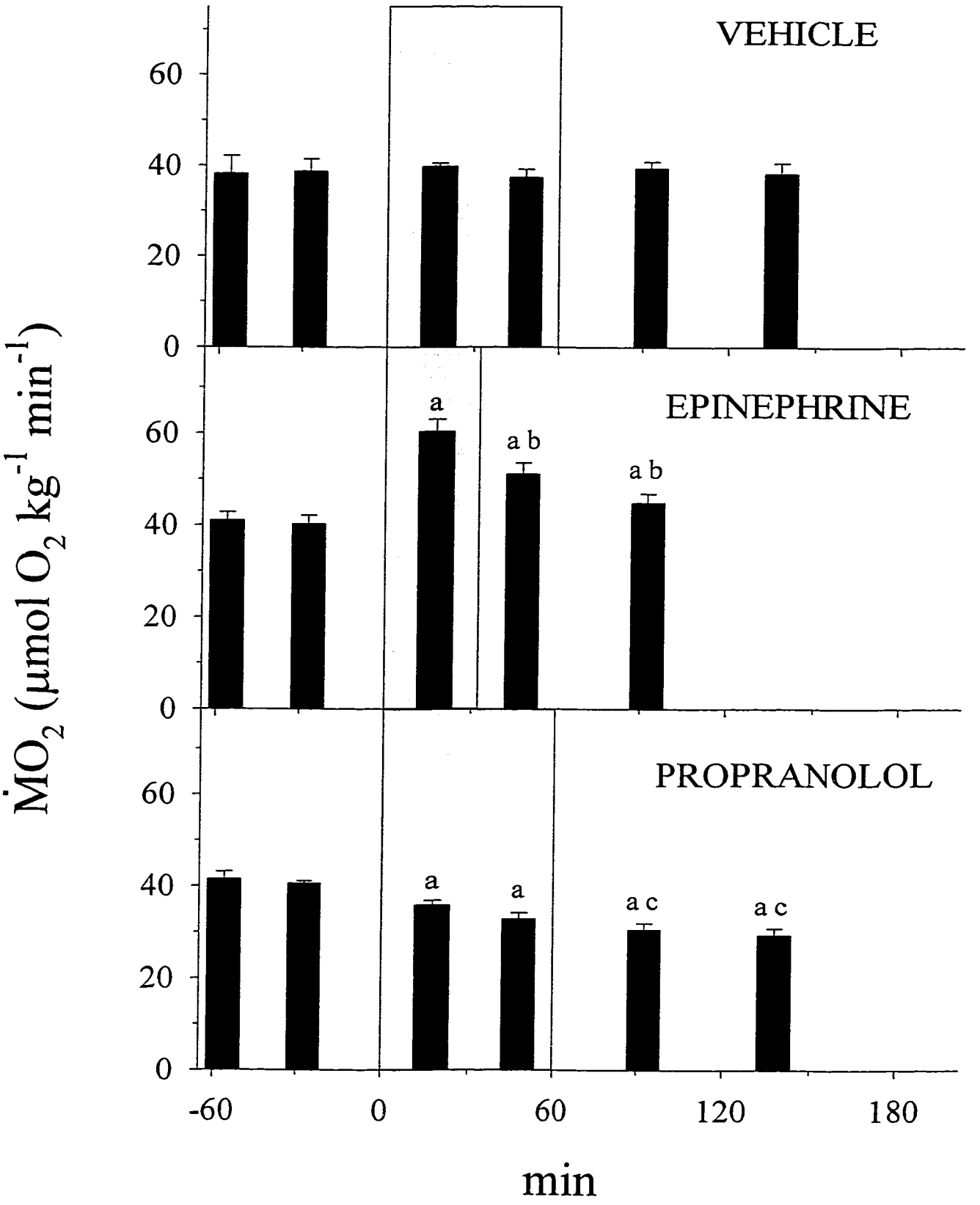

VEHICLE
EPINEPHRINE
PROPRANOLOL
$\dot{M}O_2$ (μmol O_2 kg^{-1} min^{-1})
0
20
40
60
a
a b
a b
a
a
a c
a c
-60
0
60
120
180
min

Figure 3.2 Effects of vehicle saline infusion on plasma glucose concentration (A), glucose specific activity (B), and R_a glucose (C) in rainbow trout. Shaded area indicates saline infusion and the values are means ± SEM (N=3).

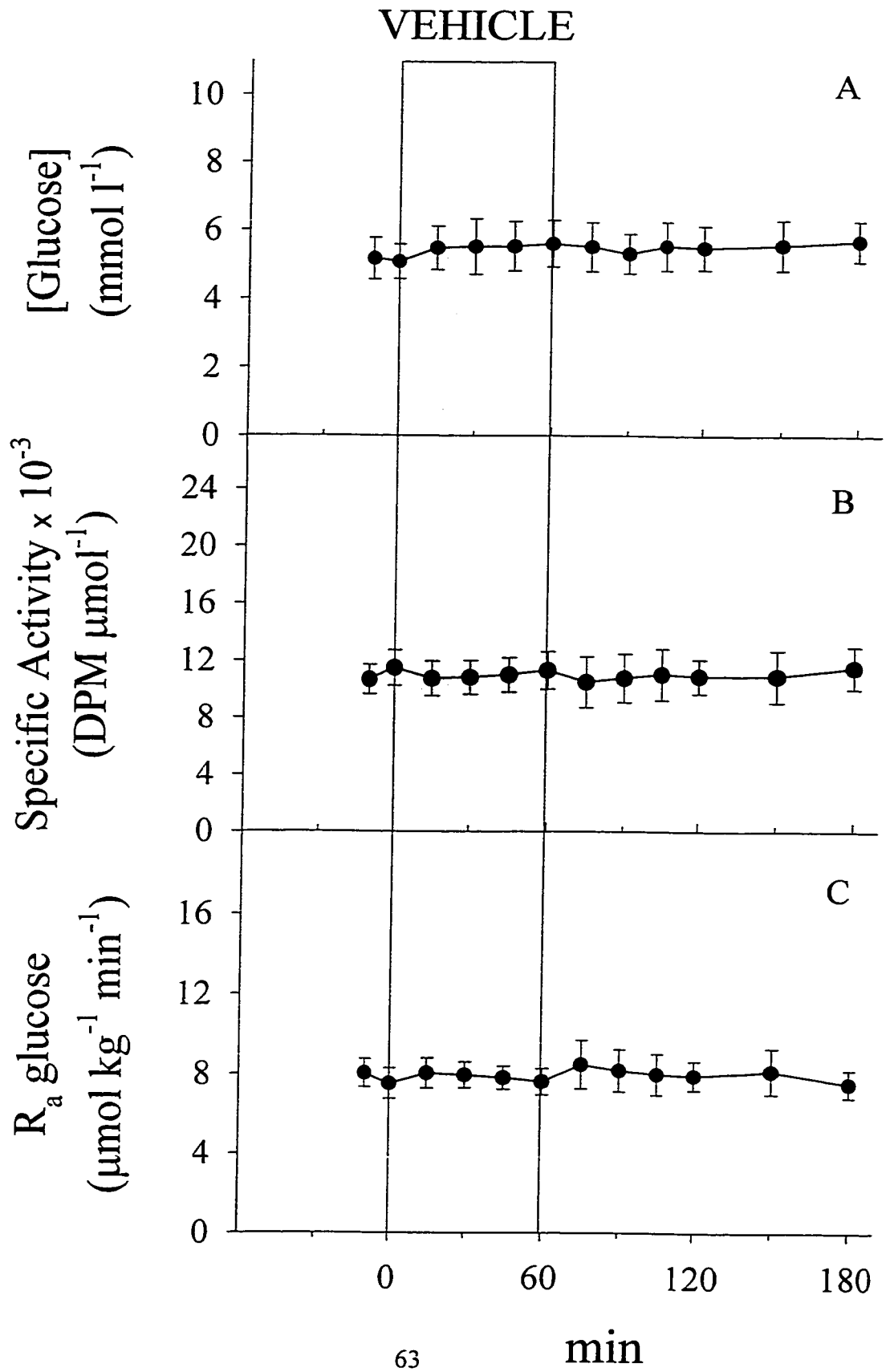
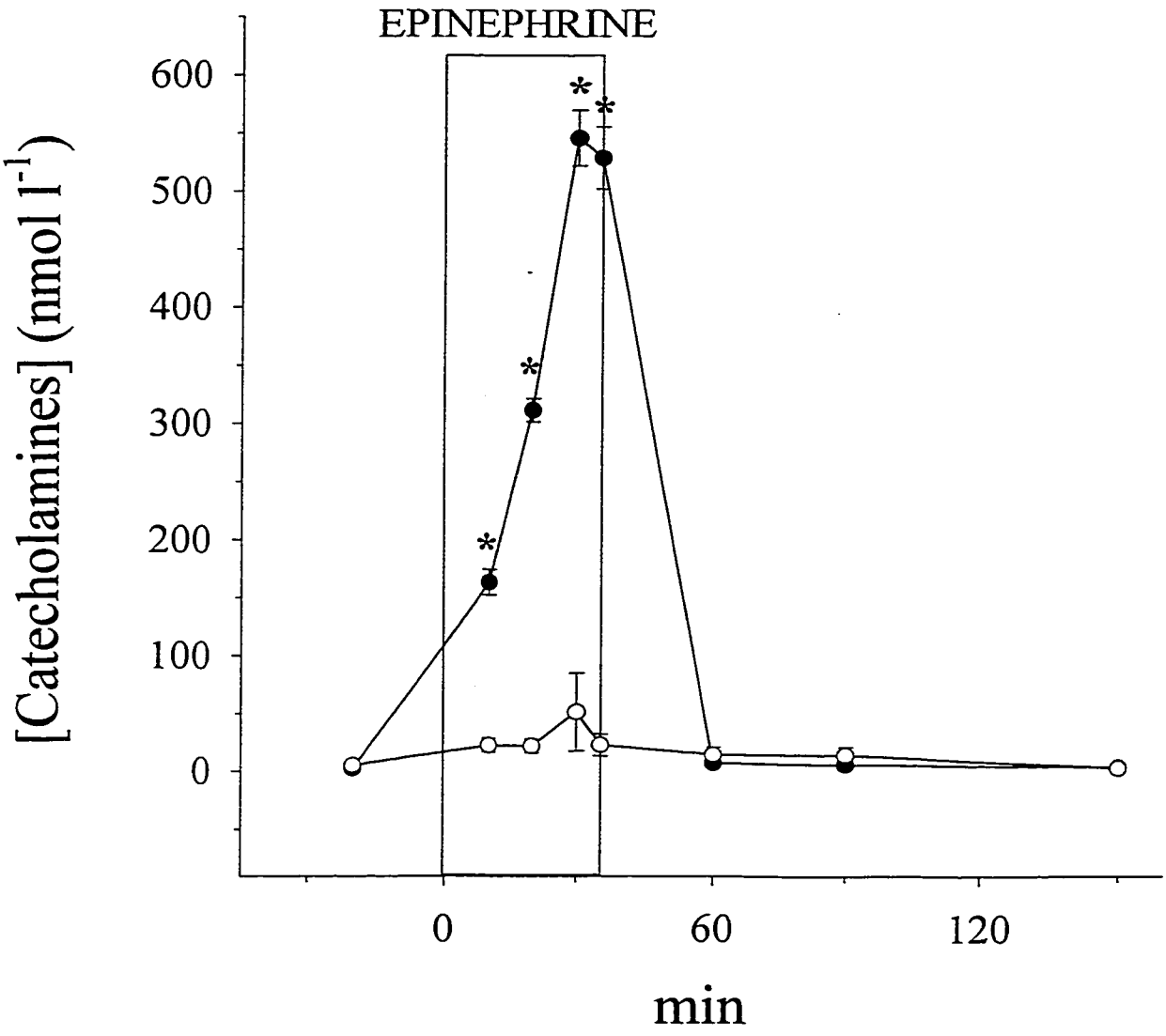

VEHICLE
A
B
C
[Glucose] (mmol l-1)
Specific Activity x 10-3 (DPM µmol-1)
Ra glucose (µmol kg-1 min-1)
10
8
6
4
2
0
24
20
16
12
8
4
0
16
12
8
4
0
0
60
120
180
min

Figure 3.3 Plasma concentration of epinephrine (closed circles) and norepinephrine (open circles) in rainbow trout. Shaded area indicates exogenous epinephrine infusion, and values are means ± SEM (N=6). * denote values significantly different from baseline levels ($P<0.05$). The sample size for 30 min and 150 min N=5.


EPINEPHRINE
[Catecholamines] (nmol l-1)
600
500
400
300
200
100
0
0
60
120
min
*
*
*
*

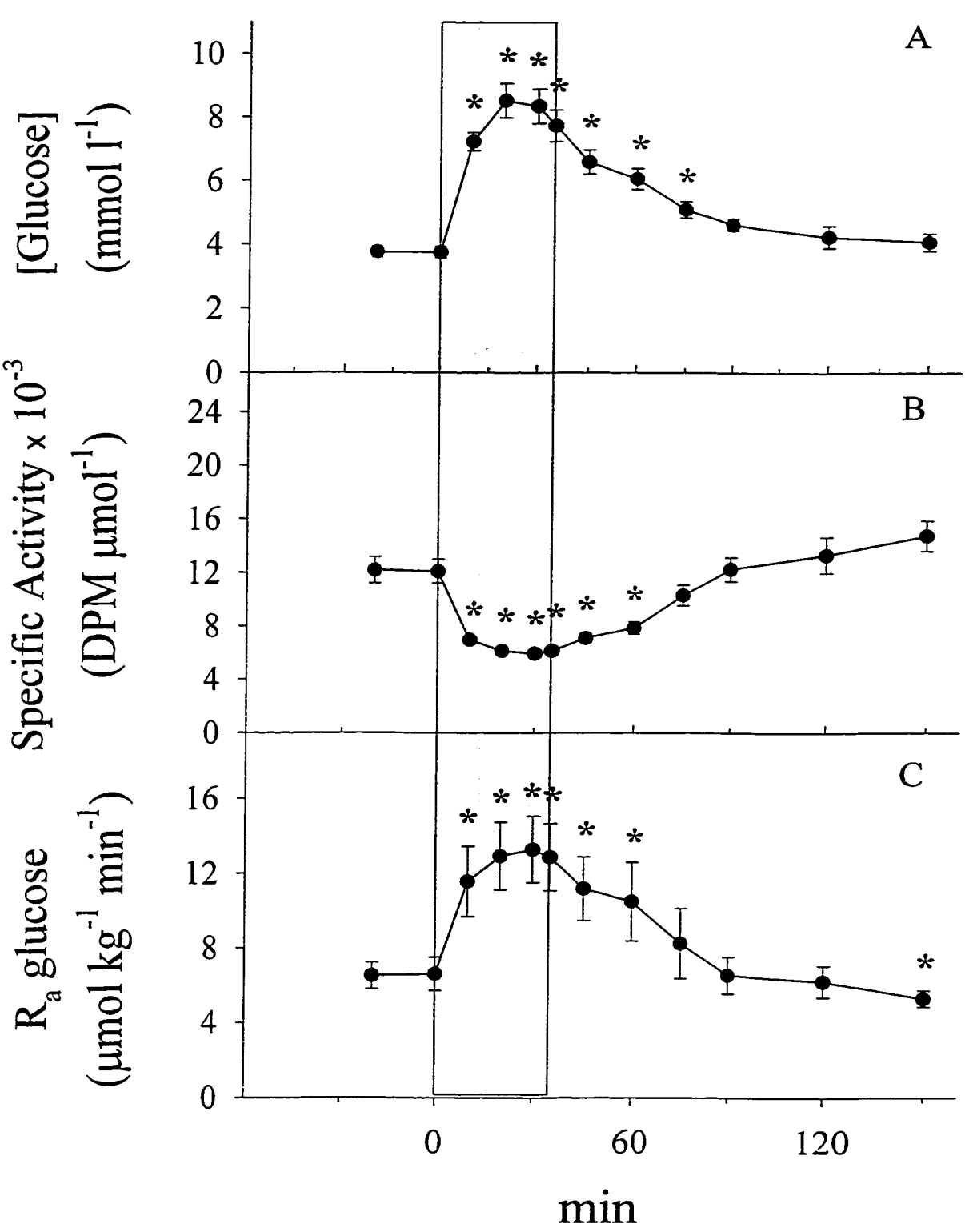
concentration, glucose specific activity, and R_a glucose are presented in Fig. 3.4. Before EPI infusion, plasma glucose concentration and R_a glucose averaged 3.75 ± 0.16 mM and 6.57 ± 0.79 μmol kg^{-1} min^{-1} (N=7), respectively. Glucose concentration increased to a maximal value of 8.51 ± 0.54 mM ($P<0.001$) after 20 min of EPI infusion, but had returned to baseline within 1 h after the end of EPI infusion (Fig. 3.4A). The administration of EPI caused a two-fold increase in R_a glucose to a maximal value of 13.30 ± 1.78 μmol kg^{-1} min^{-1} ($P<0.001$, Fig. 3.4C). Glucose production declined progressively after the end of EPI infusion. It reached baseline values after 1 h of recovery and was 5.29 ± 0.43 μmol kg^{-1} min^{-1} after 2 h of recovery, a significantly lower rate than before EPI infusion ($P<0.05$). Mean hematocrit did not change significantly during the EPI infusion experiments and averaged 24.5 ± 0.9 % (N=7).

Propranolol infusion

One hour infusion of the β-AR blocker PROP caused a decline in $\dot{M}O_2$ from a baseline value of 41.06 ± 0.96 to 32.80 ± 1.37 μmol O_2 kg^{-1} min^{-1} (N=7, $P<0.05$). A further decrease to 29.36 ± 1.47 μmol O_2 kg^{-1} min^{-1} was observed after the end of the PROP infusion (Fig. 3.1C).

Changes in glucose concentration, specific activity and glucose flux before, during and after PROP infusion are plotted in Fig. 3.5. Baseline glucose concentration and

Figure 3.4 Effects of exogenous epinephrine infusion on plasma glucose concentration (A), glucose specific activity (B), and R_a glucose (C) in rainbow trout. Shaded area indicates actual infusion time for epinephrine and values are means ± SEM (N=7). * denotes significant differences from baseline ($P<0.05$).


EPINEPHRINE
A
B
C
[Glucose] (mmol l-1)
Specific Activity x 10-3 (DPM µmol-1)
Ra glucose (µmol kg-1 min-1)
0
2
4
6
8
10
12
16
20
24
0
60
120
min

R_a glucose before PROP infusion was 4.98 ± 0.49 mmol l^{-1} (N=10) and 7.65 ± 0.92 μmol kg^{-1} min^{-1} (N=10), respectively. Plasma glucose concentration decreased progressively to reach values significantly lower than baseline in the recovery period (Fig. 3.5A). R_a glucose declined progressively to 5.17 ± 0.54 μmol kg^{-1} min^{-1} (N=10; $P<0.05$) during PROP infusion and reached a minimal value of 4.10 ± 0.56 μmol kg^{-1} min^{-1} ($P<0.05$) by the end of recovery (Fig. 3.5C). Hematocrit did not change significantly during the PROP infusion experiments and averaged 23.9 ± 0.8 % (N=10). A comparison of the relative effects of vehicle saline, EPI and PROP infusions is presented in Fig. 3.6 where percent changes in R_a glucose are quantified.

In vitro experiments

Effects of epinephrine and propranolol on glucose production by hepatocytes

Mean glucose production rates of isolated hepatocytes incubated for 60 min with or without EPI and/or PROP are summarized in Table 3.2. In each experiment, all treatment groups measured before starting the incubations (time 0) produced glucose at the same rate (experiment 1, P=0.63; experiment 2, P=0.95). Over time, the control groups of experiments 1 and 2 were not different from each other ($P>0.05$). Low and high EPI concentrations stimulated glucose production in a dose-dependent manner, but these effects were abolished by PROP (Table 3.2). Figure 3.7 summarizes the relative changes in glucose production measured after 60 min of incubation. Low EPI caused a 23% increase in glucose release ($P<0.05$, Fig. 3.7B), whereas high EPI stimulated glucose

Figure 3.5 Effects of propranolol infusion on plasma glucose concentration (A), glucose specific activity (B), and R_a glucose (C) in rainbow trout. Shaded area indicates actual infusion time of propranolol and values are means ± SEM (N=10). * denotes significant differences from baseline ($P<0.05$).

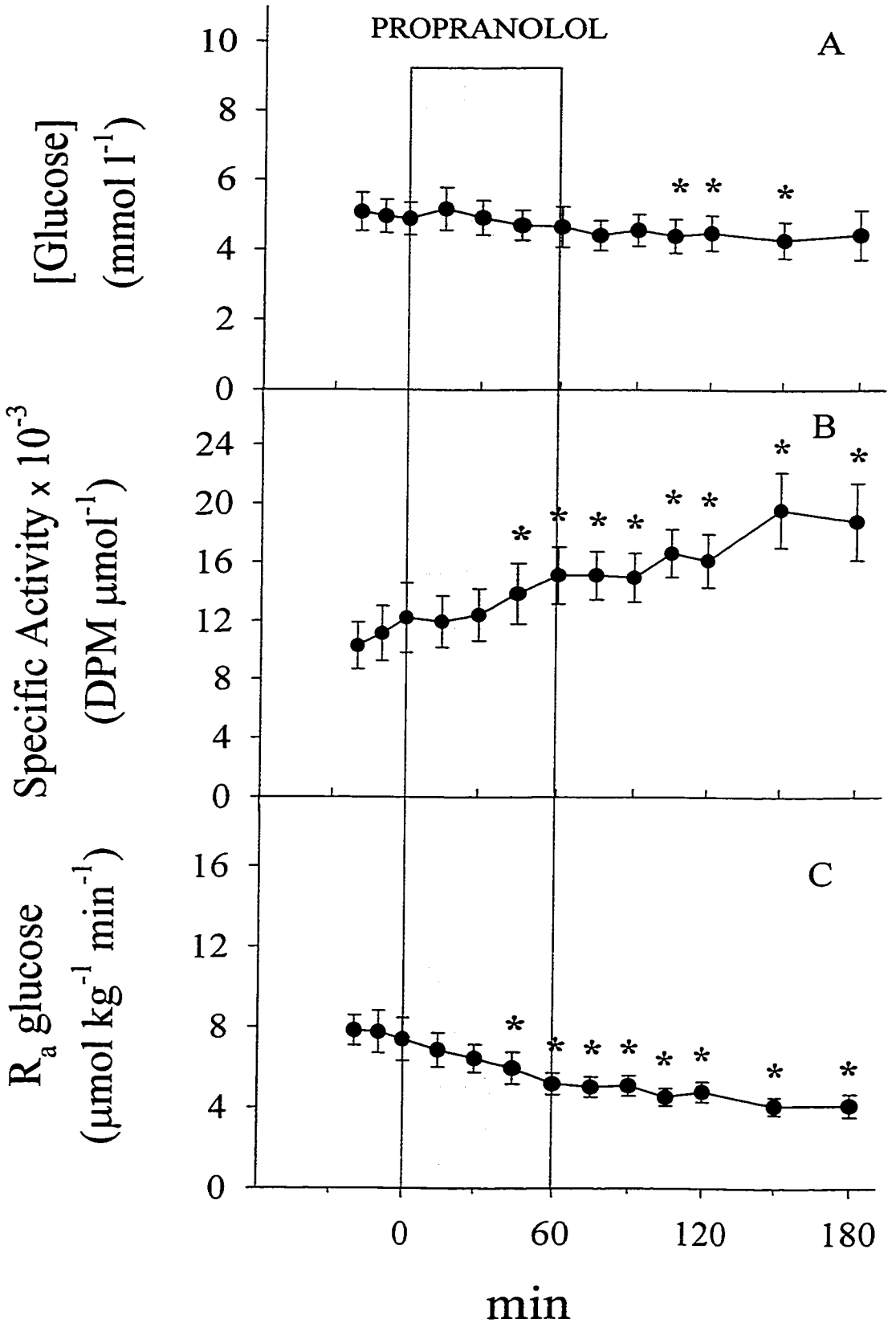

PROPRANOLOL
A
B
C
[Glucose] (mmol l-1)
Specific Activity x 10-3 (DPM µmol-1)
Ra glucose (µmol kg-1 min-1)
10
8
6
4
2
0
24
20
16
12
8
4
0
16
12
8
4
0
0
60
120
180
min

Figure 3.6 Relative effects of vehicle saline (control), epinephrine or propranolol infusion on hepatic glucose production in rainbow trout. Solid bars indicate infusion times for epinephrine and propranolol. The values are means ± SEM (controls N=3; epinephrine N=7; propranolol N=10).

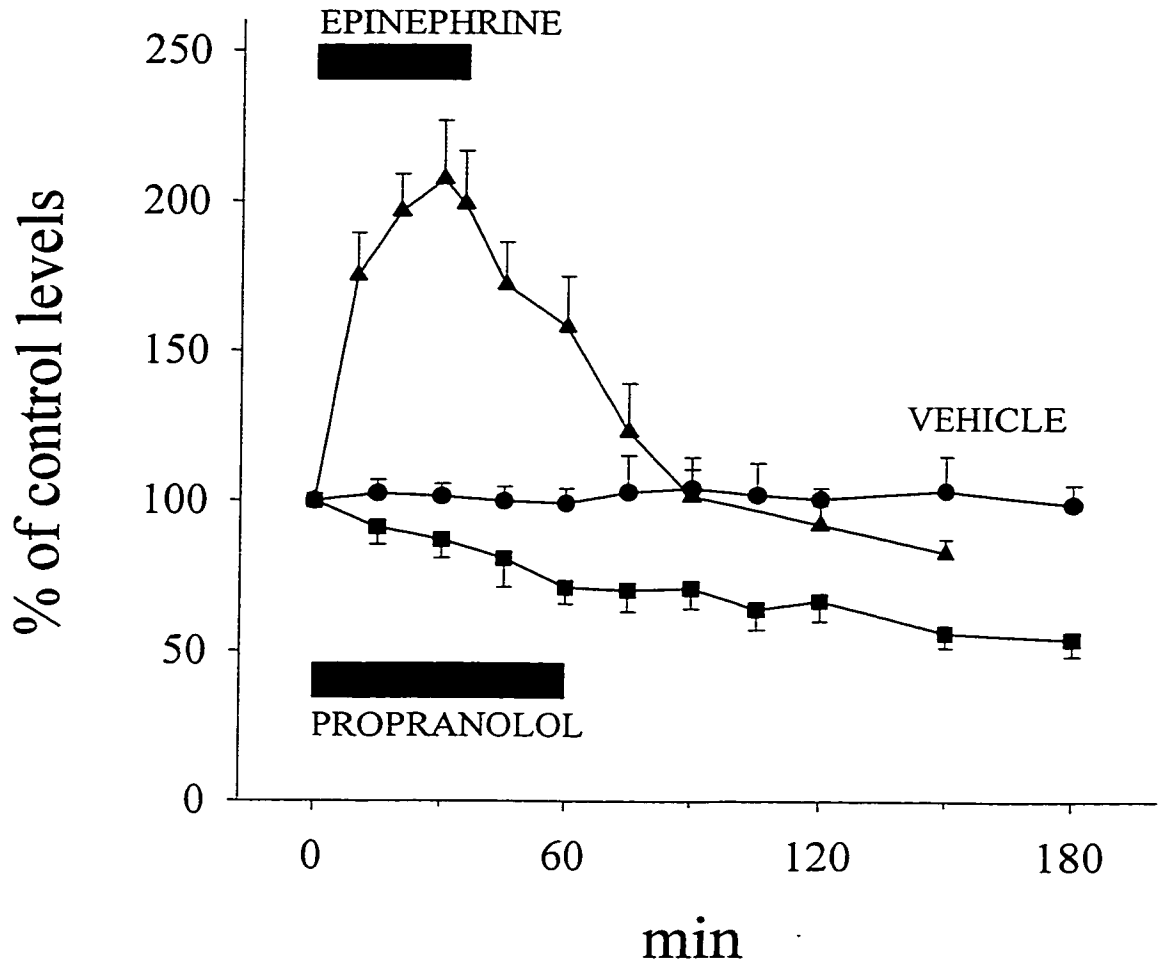
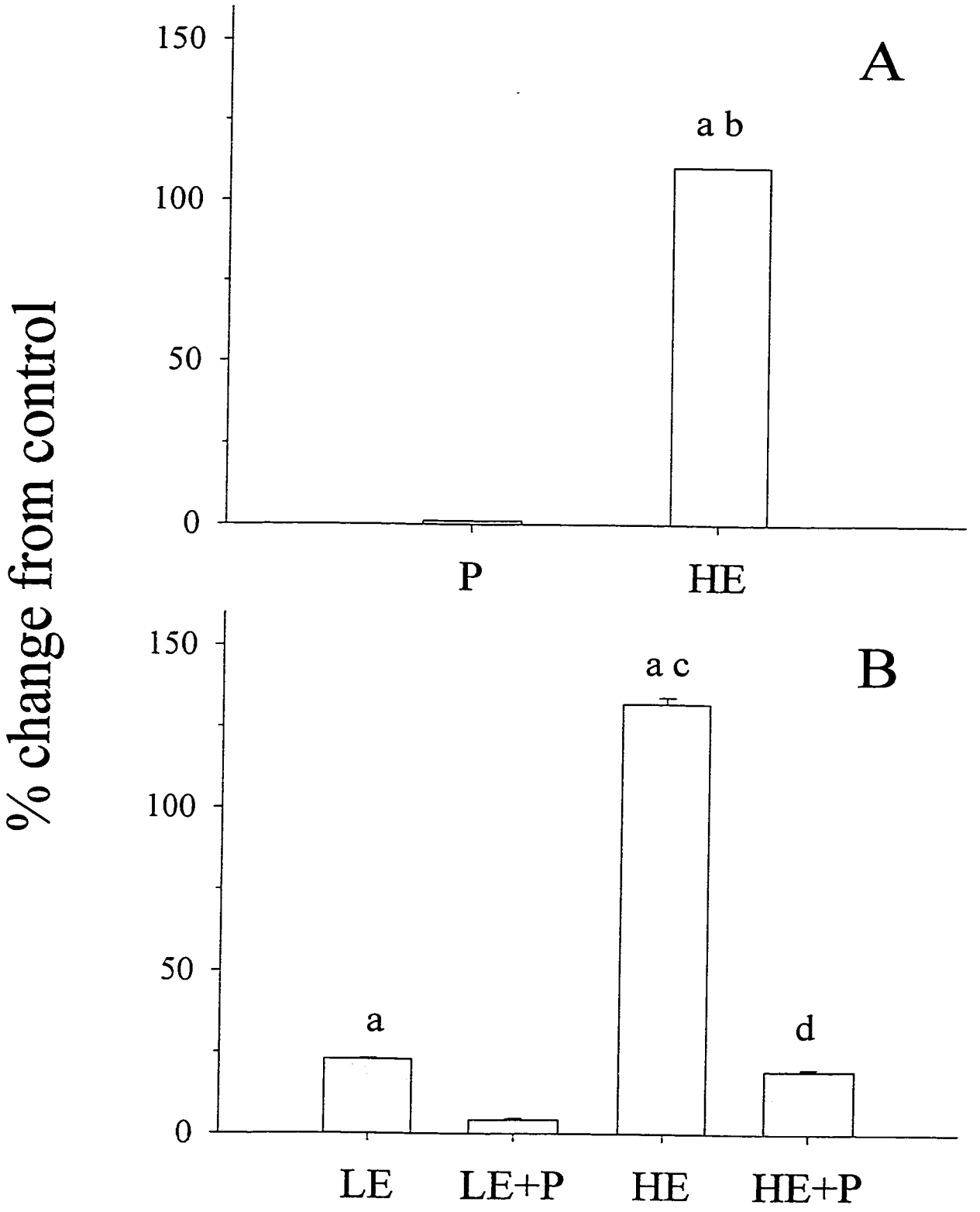

EPINEPHRINE
250
200
150
100
50
0
% of control levels
VEHICLE
PROPRANOLOL
0
60
120
180
min

Table 3.2 Amount of glucose (in μmol per g cells) released by isolated hepatocytes incubated in different media. Low EPI, 5 nM epinephrine; high EPI, 500 nM epinephrine; PROP, 50 mM propranolol. Values are means ± SEM (N=11). Letters indicate significant differences between treatments after 60 min of incubation ($P<0.05$): a different from control, b different from PROP, c different from low EPI, and d different from high EPI.

Time	Control (-15 min)	15 min	30 min	45 min	60 min	Sig
Experiment 1						
Control	6.5±0.7	6.7±0.8	12.6±1.7	18.6±2.2	20.6±2.2	
PROP 50 mmol l^{-1}	6.5±0.7	6.2±0.9	11.8±1.4	16.9±2.1	22.8±2.6	
EPI 500 nmol l^{-1}	6.4±0.6	12.2±1.4	23.8±2.9	34.4±4.3	44.1±5.6	a b
Experiment 2						
Control	4.4±0.2	4.4±0.2	8.8±0.4	13.4±0.6	17.5+0.8	
EPI 5 nmol l^{-1}	4.4±0.2	5.5±0.3	10.5±0.6	15.8±0.9	21.4±1.1	a
EPI + PROP 5 nmol l^{-1}	4.6±0.2	4.6±0.3	8.7±0.6	13.7±0.9	18.2±1.1	
Epinephrine 500 nmol l^{-1}	4.4±0.2	9.9±0.8	19.2±1.1	27.2±1.6	35.9±2.1	a c
Epi + Prop 500 nmol l^{-1}	4.4±0.2	5.1±0.5	10.5±0.7	15.6±0.9	20.7±1.3	d

Figure 3.7 Relative changes in total glucose release from isolated rainbow trout hepatocytes after 60 min of incubation with 50 mM propranolol (P), 5 mM epinephrine (LE) or 540 mM epinephrine (HE). Two series of incubations were carried out: experiment 1 (A) had control, P and HE-treated cells; experiment 2 (B) had control, LE, LE+P, HE and HE+P-treated cells. Significant differences ($P<0.05$) are indicated by letters: a different from control, b different from propranolol (P), c different from low epinephrine (LE) and d different from high epinephrine (HE).


% change from control
150
100
50
0
A
a b
P
HE
150
100
50
0
B
a c
a
d
LE
LE+P
HE
HE+P

output by 110% and 133% in experiments 1 and 2, respectively ($P<0.001$, Fig. 3.7A and B). PROP had no effect in the absence of EPI (Fig. 3.7A), and it prevented both low, and high EPI from stimulating glucose production (Fig. 3.7B).

Comparison of in vivo and in vitro glucose production rates

Figure 3.8 shows a comparison of R_a glucose measured in the whole organism and in isolated hepatocytes. Rates of glucose production measured *in vitro* in μmol per g hepatocytes (Table 3.2) were converted to μmol min^{-1} per kg body mass to compare them with baseline R_a glucose measured *in vivo* that averaged 7.31 ± 0.54 μmol kg^{-1} min^{-1} (N=20) after correction for renal glucose production (see Methods). Mean *in vitro* R_a glucose was 3.13 ± 0.09 μmol kg^{-1} min^{-1} (N=22), and increased to 3.83 ± 0.16 (N=11) and 5.92 ± 0.19 μmol kg^{-1} min^{-1} (N=22) in the presence of low (5 nM) and high (500 nM) EPI concentration, respectively ($P<0.05$). The relative effects of EPI and PROP *in vivo* and *in vitro* are compared in Fig. 3.9. High EPI concentration had the same effect *in vivo* and *in vitro*, stimulating R_a glucose by 102% and 89%, respectively. PROP alone caused a 46% decline in R_a glucose *in vivo*, but had no significant effect *in vitro* (+1.8%).

Figure 3.8 Rates of glucose production (R_a glucose) of rainbow trout in μmol min^{-1} per kg body mass measured *in vivo* and in isolated hepatocytes. Values are means ± SEM. C=control *in vitro* values (without epinephrine or propranolol in the incubation medium) and all other abbreviations as in Fig. 3.7. Significant differences ($P<0.05$) are indicated by letters: a different from *in vivo*, b different from *in vitro* control, c different from LE, d different from LE+P, and e different from HE.

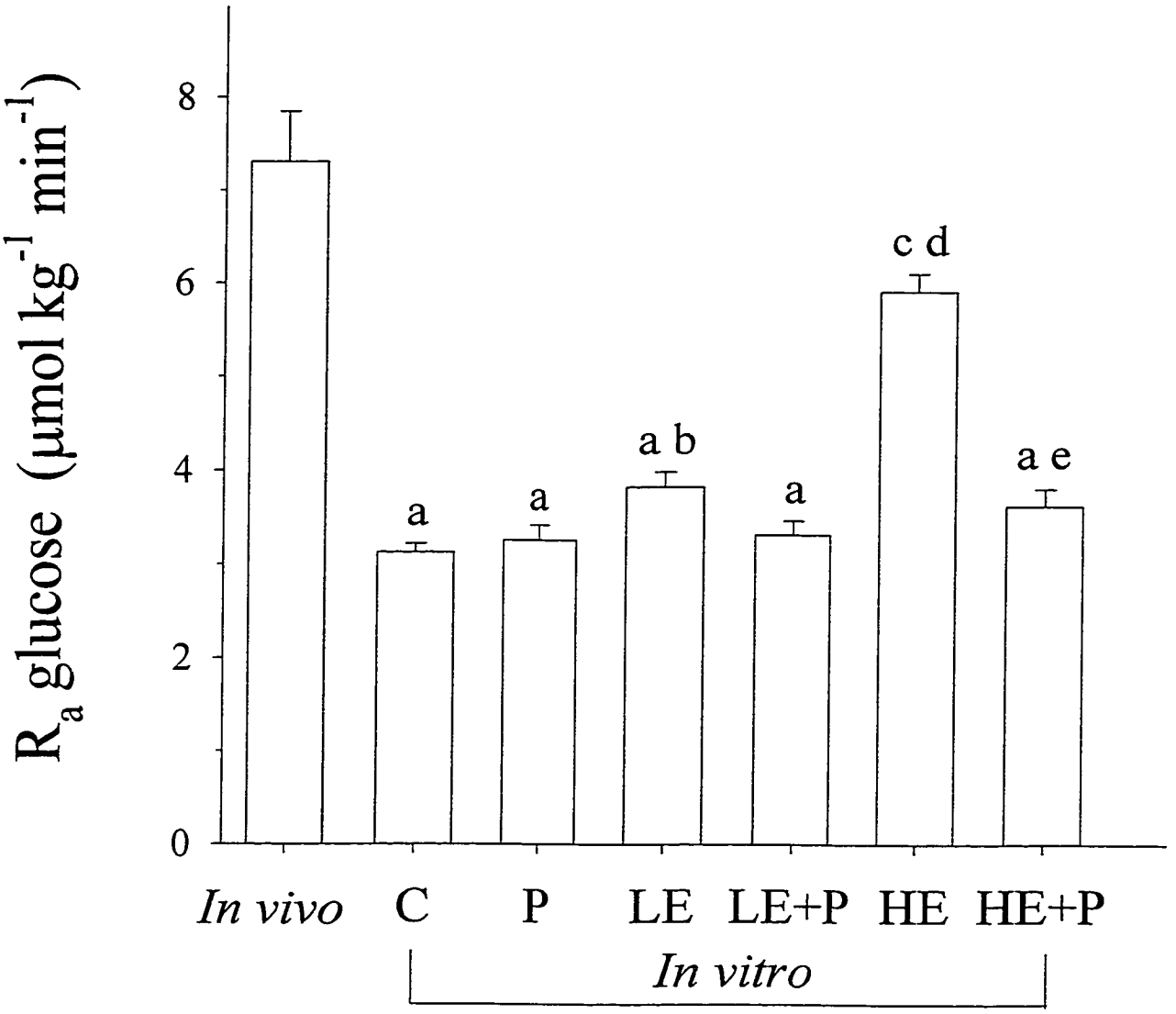
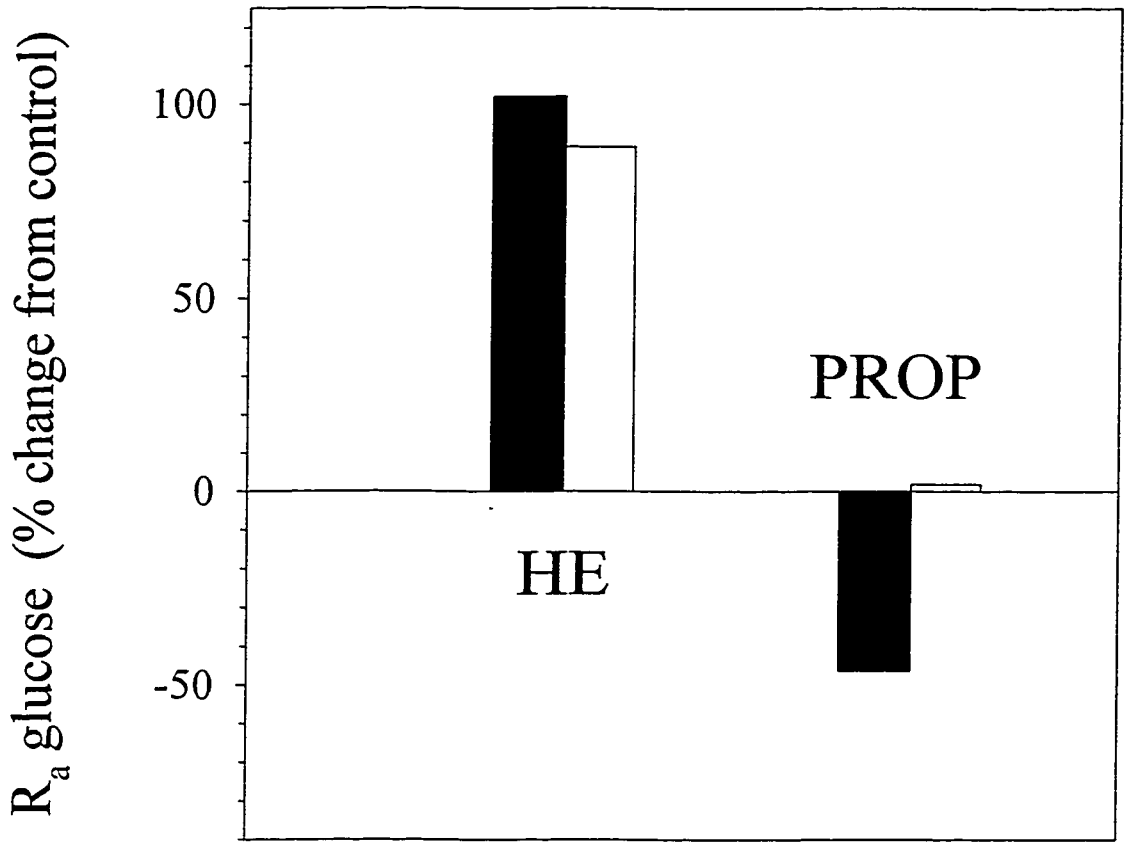

R_a glucose (μmol kg^{-1} min^{-1})
8
6
4
2
0
a
a
a b
a
c d
a e
In vivo
C
P
LE
LE+P
HE
HE+P
In vitro

Figure 3.9 Relative effects of epinephrine and propranolol on hepatic glucose production of rainbow trout measured *in vivo* (solid bars) and in isolated hepatocytes (open bars). The first open bar represents change in R_a glucose from control when hepatocytes are incubated with propranolol alone and the second open bar represents change in R_a glucose from control value when hepatocytes are exposed to propranolol in presence of low epinephrine (5 nmol l^{-1}).


R_a glucose (% change from control)
100
50
0
-50
HE
PROP

Discussion

Earlier *in vivo* measurements of fish substrate kinetics had revealed high basal rates of hepatic glucose production and a limited ability to increase R_a glucose (Haman *et al.*, 1997a; Haman and Weber, 1996; Haman *et al.*, 1997b; Shanghavi and Weber, 1999). Stresses such as moderate exercise (Shanghavi and Weber, 1999) and acute changes in water temperature (Haman *et al.*, 1997b) were shown to depress glucose production, suggesting that resting unstressed fish already release glucose at close to maximal rates. Results from the present study demonstrate otherwise and provide a new minimal estimate of reserve capacity for *in vivo* glucose production. Except for a small and transient increase in R_a observed during acute hypoxia (Haman *et al.*, 1997b), this is the first example where fish strongly stimulate hepatic glucose production *in vivo*, in this case, responding to a hormonal signal. Elevated plasma EPI levels (Fig. 3.3) caused a 2-fold increase in R_a glucose (Figs. 3.4 and 3.6), whereas previous experiments by others showed that cortisol (Andersen *et al.*, 1991; Vijayan and Moon, 1994) and estrogen (Washburn *et al.*, 1992) had no significant effects.

Mechanisms of epinephrine action on hepatic glucose production

Arterial infusion of EPI caused hyperglycemia (Fig. 3.4A) as noted previously in the same species (Morata *et al.*, 1982), in other teleosts (Ottolenghi *et al.*, 1984; Van Raaij *et al.*, 1995), and in humans (Sacca *et al.*, 1983). Even though the observed increase in blood glucose had to be caused by a temporary mismatch between R_a and R_d, the difference between these rates could not be detected statistically because R_d increased

almost simultaneously with R_a. Therefore, hyperglycemia resulted from a rapid increase in R_a, whereas the EPI-induced reduction in R_d known to occur in mammals (Cherrington, 1994) did not play a role in fish. The increase in trout R_a glucose could be mediated through the direct activation of glycogenolysis and gluconeogenesis, and via indirect mechanisms involving changes in hepatic blood flow or in the levels of other circulating hormones such as glucagon, insulin, and cortisol. The respective contributions of these different factors cannot be determined from our results and further work will be needed to evaluate their potential impact separately.

In mammals, the initial increase in R_a glucose after EPI administration is caused by the immediate stimulation of glycogenolysis, whereas gluconeogenesis is only activated later when glycogen stores are somewhat depleted (Cherrington, 1994; Sacca *et al.*, 1983; Stevenson *et al.*, 1991). *In vitro* experiments on various species of fish show that EPI increases glycogenolysis by activating glycogen phosphorylase (Janssens and Lowry, 1987; Wright et al., 1989). In isolated fish hepatocytes, glucose production is supported almost exclusively through the breakdown of glycogen whereas gluconeogenesis only plays a minor role. However, this overwhelming dominance of glycogenolysis may be an artefact of the cell isolation procedure and gluconeogenesis may be more significant *in vivo* (Mommsen *et al.*, 1988). High EPI levels are known to depress pyruvate kinase activity in fish liver, thereby causing a reciprocal increase in gluconeogenesis and decrease in glycolysis (Mommsen *et al.*, 1988; Wright *et al.*, 1989). Furthermore, EPI may cause the peripheral mobilization of several gluconeogenic precursors and promote their uptake by the liver, as in mammals (Sacca *et al.*, 1983; Stevenson *et al.*, 1991).

Role of epinephrine in maintaining basal glucose production

Resting EPI levels are not responsible for sustaining basal glucose production in mammals (Cherrington, 1994) but our study shows that they can play a significant role in fish. When the β-adrenergic effects of resting EPI were blocked by the infusion of PROP *in vivo*, plasma glucose concentration and R_a glucose of trout decreased by 14% and 46%, respectively (Figs. 3.5 and 3.6). Furthermore, glucose production by trout hepatocytes incubated at basal EPI concentrations showed a 20% decline when PROP was added to the medium (Fig. 3.7). Taken together, these results suggest that, in the resting state, EPI is present at higher concentrations in the portal circulation of fish than mammals, and the observation that teleosts have much higher resting arterial EPI levels than mammals (Randall and Perry, 1992) supports this view. The absence of a decrease in the R_a glucose of mammals after the administration of α or β-blockers has been attributed to the fact that their portal concentrations of catecholamines are negligible under basal conditions (Cherrington, 1994).

Glucose production rates: in vivo vs in vitro

After standardizing diet, temperature and body size, isolated trout hepatocytes incubated without hormones still produce glucose at less than half the basal rate observed *in vivo* (3.13 vs 7.31 μmol kg^{-1} min^{-1}; Fig. 3.8). This difference is due to a combination of factors that may include hormonal and neural effects only acting *in vivo*, as well as structural and/or functional damage incurred by the cells during isolation. Resting arterial EPI levels are responsible for a fraction of the difference because the addition of 5 nM

EPI to the incubation medium increases glucose production *in vitro* (Figs. 3.7B and 3.8), and PROP causes a decrease in R_a glucose *in vivo* (Fig. 3.5) as well as in isolated cells incubated with basal EPI levels (Fig. 3.7). Isolated cells only approached *in vivo* rates when they were incubated with 500 nM EPI, a concentration simulating extreme arterial conditions after exhaustive exercise (Fig. 3.8). Therefore, other factors than basal EPI are also responsible for the 2-fold difference between basal glucose production rates observed *in vivo* and *in vitro*. Resting levels of glucagon, norepinephrine, and cortisol, as well as direct sympathetic stimulation may be involved in maintaining basal R_a glucose *in vivo*. However, further experiments will be needed to clarify this issue. The mean basal rate of glucose production measured here *in vitro* (3.13 $\mu mol\ kg^{-1}\ min^{-1}$) falls within the range of values previously obtained by others for trout hepatocytes (1-5 $\mu mol\ kg^{-1}\ min^{-1}$, see methods for conversion to this unit) (Dugan and Moon, 1998; Mommsen, 1986; Mommsen and Moon, 1990; Mommsen *et al.*, 1988). We could only find one *in vitro* study reporting glucose production rates as high as measured *in vivo* (Morata *et al.*, 1982). Interestingly, these high *in vitro* values were obtained in experiments carried out on liver slices rather than isolated cells, suggesting that disrupting the integrity of the liver tissue impairs normal glucose production.

In humans, EPI also stimulates R_a glucose indirectly by increasing hepatic blood flow and by activating glucagon secretion which enhances liver gluconeogenesis (Sacca *et al.*, 1983). Here, high EPI concentration had similar relative effects *in vivo* and *in vitro*, causing baseline R_a glucose to double in both cases. These findings suggest that the indirect pathways of EPI stimulation acting *in vivo* in mammals do not play a significant role in trout.

β- vs α-adrenergic effects of epinephrine

Both *in vivo* and *in vitro* experiments show that the stimulating effect of EPI on the R_a glucose of rainbow trout is eliminated in the presence of PROP. The signal transduction pathway involving the binding of EPI to β-adrenoreceptors, the activation of adenyl cyclase, and the up-regulation of glycogen phosphorylase *a* have been well characterized in fish (Fabbri *et al.*, 1998). The reverse phenomenon has been observed when treatment with PROP caused a 3 to 5-fold decrease in glycogen phosphorylase activity (Wright *et al.*, 1989). Recently, the presence of α-adrenoreceptors has been demonstrated in the membrane of rainbow trout hepatocytes, but no clear functional link with glucose metabolism has yet been established (Fabbri *et al.*, 1995). Taken together, these results show that circulating EPI stimulates trout hepatic glucose production mainly via β-adrenoreceptors and that α-receptors could only play a minor role if any.

Effects of epinephrine on glucose disappearance

Because the rates of glucose production (R_a) and glucose disposal (R_d) were never statistically different, all the figures showing changes in R_a also depict how R_d was affected. Arterial EPI infusion caused R_d glucose to double (Fig. 3.4), and two possible mechanisms for this response can be proposed: i) a simple mass action effect mediated by hyperglycemia and/or ii) the activation of the glucose transporter (GLUT-4). The large augmentation in plasma glucose levels (Fig. 3.4A) expanded the diffusional concentration gradient driving glucose into cells and this mechanism must be partly responsible for the

observed increase in R_d glucose. However, an increase in the rate of glucose transport through the translocation of GLUT-4 to the cell membrane, as observed in mammals, is unlikely to be involved in the EPI stimulation of R_d in fish. To date, every attempt to demonstrate the presence of GLUT-4 in fish tissues has failed, with the possible exception of pancreatic cells in *Tilapia* (Wright *et al.*, 1998).

Conclusions

By combining results from our *in vivo* and *in vitro* experiments, the following conclusions can be drawn from this study: i) The EPI-induced hyperglycemic response of rainbow trout is caused by the stimulation of R_a glucose, but the concomitant reduction in R_d glucose observed in mammals does not occur in trout. ii) Basal levels of circulating EPI are partly responsible for maintaining resting R_a glucose in rainbow trout. iii) EPI stimulates hepatic glucose production in a dose-dependent manner and its effects are mainly mediated by β-adrenoreceptors. iv) Isolated trout hepatocytes produce glucose at about half the basal rates measured *in vivo*, even when diet, temperature and body size are standardized, and basal arterial EPI is partly responsible for this discrepancy, v) the relative increases in R_a glucose after EPI stimulation are similar *in vivo* and *in vitro*, suggesting that indirect *in vivo* effects of EPI such sympathetic stimulation, changes in hepatic blood flow, or changes in the levels of other circulating hormones do not play an important role in trout.

CHAPTER 4

GENERAL CONCLUSIONS

This thesis has examined the effects of sustainable exercise and circulating EPI on hepatic glucose production in live rainbow trout. Contrary to expectation, rainbow trout do not increase hepatic glucose production during sustained exercise. In fact, the first part of this thesis shows that prolonged exercise causes a 30% decline in R_a glucose and that circulating EPI may be partly responsible for this response. Such a decline is rather unexpected because all other animals measured to date (i.e. mammalian species only) show a 2 to 4–fold increase at comparable exercise intensities. The decline in R_a glucose observed in trout suggests that circulating glucose is probably not an important metabolic fuel for prolonged submaximal swimming in trout. Furthermore, my study is the first to show that rainbow trout have the capacity to regulate circulating glucose concentration throughout prolonged exercise even though they are considered to be poor glucoregulators.

The second part of this thesis examined the effects of circulating EPI concentrations on R_a glucose by elevating EPI concentration through exogenous infusion or by blocking some of its effects with the β-blocker propranolol. *In vivo* experiments (continuous tracer infusions in live animals) and *in vitro* experiments (isolated hepatocytes incubated in different media) were combined to investigate the regulation of glucose production by rainbow trout liver. Results allow to conclude that trout have the capability of increasing hepatic glucose production by more than two fold over resting values. Unlike mammals, the observed EPI-induced hyperglycemia of trout is entirely caused by the stimulation of R_a glucose because R_d glucose is not reduced by this hormone in fish. Basal circulating levels of EPI are involved in maintaining resting R_a glucose. EPI stimulates *in vitro* glucose production in a dose-dependent manner and its

effects are mainly mediated by β-adrenoreceptors. Isolated trout hepatocytes produce glucose at half the basal rate measured *in vivo*, even when diet, temperature and body size are standardized, and basal circulating EPI is responsible for part of this discrepancy. The relative increase in R_a glucose after EPI stimulation is similar *in vivo* and *in vitro*, suggesting that indirect *in vivo* effects of EPI such as changes in hepatic blood flow or in other circulating hormones do not play an important role in the regulation of glucose production in trout.

The regulation of hepatic glucose production during aerobic swimming in fish is of particular interest. It could be further investigated following a protocol similar to the one used in this study, but at various swimming intensities and duration. In order to establish the precise role of EPI on hepatic glucose production, the quantification of R_a glucose by direct infusion of EPI into the portal vein should be conducted. These studies should include hormonal clamping, especially of insulin and glucagon, to be able to remove their effects on R_a glucose and focus on pure EPI-induced changes in flux. Moreover, future studies should examine the changes in R_a glucose during a longer exposure to elevated EPI concentrations. More *in vitro* and *in vivo* experiments are also needed using α-AR antagonists to establish the role of these receptors on R_a glucose. Certainly, fish carbohydrates metabolism and its regulation is a rich field for further investigation.

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