EFFECTS OF ACUTE HYPOXIA AND COLD EXPOSURE ON GLUCOSE AND FATTY ACID FLUXES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

François Haman

Thèse soumise à

l'Ecole des études supérieures et de la recherche

Université d'Ottawa

en vue de l'obtention de la maîtrise ès sciences à

L'institut de biologie d'Ottawa-Carleton

Thesis submitted to the

School of Graduate Studies and Research

University of Ottawa

in partial fulfillment of the requirements for the M.Sc. degree in the

Ottawa-Carleton Institute of Biology

François Haman, Ottawa, Canada, 1996.

National Library of Canada

Bibliothèque nationale du Canada

Acquisitions and Bibliographic Services Branch

Direction des acquisitions et des services bibliographiques

395 Wellington Street
Ottawa, Ontario
K1A 0N4

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-16443-8

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Master of Science (1996)
(Biology)

University of Ottawa
Université d'Ottawa

Title: Effects of acute hypoxia and cold exposure on glucose and fatty acid fluxes in rainbow trout (Oncorhynchus mykiss)

Author: François Haman, B.Sc. (Kinan), B.Ed., B.E.O.

Supervisors:
Dr. Jean-Michel Weber - Professor, Department of Biology, University of Ottawa.

EFFECTS OF ACUTE HYPOXIA AND COLD EXPOSURE ON GLUCOSE AND FATTY ACID FLUXES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

SUMMARY

The continuous isotope infusion technique is the most accurate way to measure metabolite fluxes *in vivo*, but it has never been used in trout. Therefore, the first goal of my thesis is to develop a double catheterization method allowing simultaneous infusion of labeled compounds and blood sampling, and to quantify glucose fluxes in resting rainbow trout by continuous infusion of 6-^{3}H-glucose (Chapter 2). The new cannulation technique consists of placing both sampling and infusing catethers in the dorsal aorta. Continuous isotope infusion results show that resting glucose turnover is 9.5 ± 0.8 μmol kg^{-1} min^{-1}, a value 4 to 9 times higher than most rates measured with bolus injection technique reported in the literature. Consequently, the second goal of my thesis is to quantify the accuracy of the continuous infusion technique in estimating glucose turnover rate (Chapter 3). While under anesthetic, trout are hepatectomized and liver glucose production is artificially replaced by a pump infusing unlabelled glucose at a known rate. Glucose flux measured with the continuous infusion technique using either plasma or whole blood is not significantly different from artificial pump glucose entry rate. Therefore, this result suggests that the bolus injection method widely used in previous trout studies strongly underestimates true glucose flux.

Using the continuous infusion technique developed and validated in chapters 2 and 3, respectively, my third goal is to investigate the effects of two environmental disturbances namely acute hypoxia and cold exposure, on the rates of glucose and NEFA (non-esterified fatty acids) turnover in rainbow trout. My hypotheses are that: 1) during

hypoxia, a fuel preference for carbohydrates would lead to an increase in glucose flux and a decrease in NEFA flux; 2) during cold exposure, both glucose and NEFA fluxes would decrease in proportion to metabolic rate depression. During a 90 min exposure to acute hypoxia, hyperglycaemia is associated with a transient increase in glucose appearance rate 15 minutes after reaching 25% oxygen saturation. Thereafter, glucose flux returns to normoxic levels suggesting that *in situ* utilization of tissue glycogen reserves is the main substrate for anaerobic glycolysis rather than circulatory glucose during hypoxia. As a result of hypoxia, NEFA turnover rate decreases by 52% from normoxic levels suggesting that this metabolic fuel is not a preferred substrate during hypoxia. During acute exposure to cold water (15°C to 6°C), glucose and NEFA turnover rates decrease proportionally to metabolic depression resulting in a similar Q_{10} value of approximately 2. This suggests that no fuel preference is occurring during cold exposure. However, while glucose concentration only shows a slight overall decrease, NEFA concentration increases by 30% after 100 minutes at 6°C.

Results from both hypoxia and temperature experiments show that changes in plasma metabolite concentrations indicate nothing about flux changes. This thesis demonstrates that the interpretation of changes in concentration may be misleading, and that direct measurement of fluxes is necessary to understand the dynamic impact of experimental manipulation on substrate metabolism. The double cannulation and continuous infusion techniques presented in this thesis provide the necessary tools to study *in vivo* metabolite fluxes in fish. Use of this technique can now be extended to the study of other metabolites under steady or nonsteady-state conditions such as swimming, thermal/osmotic stress, and hormonal treatment.

RESUME

Bien que la technique d'infusion continue soit la façon la plus précise de mesurer des flux de métabolites *in vivo*, elle n'a jamais été utilisée chez la truite. Par conséquent, ma thèse vise premièrement à developpper une cannulation double permettant une infusion de traceurs métaboliques et un échantillonnage simultanné, et à quantifier le flux de glucose chez une truite arc-en-ciel au repos à l'aide d'une infusion continue de 6-^{3}H-glucose (Chapter 2). Cette nouvelle technique de cannulation consiste à placer les cathéter d'infusion et d'échantillonnage dans l'aorte dorsale. Suite à une infusion continue d'isotope, le taux de renouvellement du glucose au repos a été mesuré à 9.5 ± 0.8 μmol kg^{-1} min^{-1}. Cette valeur est 4 à 9 fois plus élevée que les taux mesurés précédemment chez la truite arc-en-ciel. Conséquemment, le second but de ma thèse est d'estimer la précision de la technique d'infusion continue dans la quantification du flux du glucose (Chapter 3). Des truites anesthésiées sont hépatectomisées et la production de glucose est remplacée artificiellement par une pompe infusant à un taux connu. Les flux de glucose mesurés par infusion continue en utilisant soit du plasma soit le sang total ne sont pas significativement différents du taux de production de glucose artificiellement infusé. Par conséquent, ce résultat suggère que la méthode d'injection d'un bolus d'isotope, technique la plus utilisée chez la truite, est reliée à une importante sous-estimation du flux de glucose réel.

En utilisant la technique d'infusion continue développée et validée respectivement au chapitre 3 et 4, le but final de ma thèse est d'étudier l'effet de deux stress

environnementaux, notamment l'hypoxie aigüe et un abaissement rapide de température, sur les flux de glucose et d'acides gras non-estérifiés (NEFA) chez la truite arc-en-ciel. Mes hypothèses sont: 1) au cours de l'hypoxie, une augmentation de l'utilisation des hydrates de carbone sera liée à une augmentation du flux de glucose et une diminution du flux de NEFA; 2) au cours d'un abaissement rapide de température, les flux de glucose et de NEFA diminueront proportionnellement à la dépression du taux métabolique. Durant 90 minutes d'hypoxie aigüe, une brève augmentation du taux d'apparition du glucose 15 minutes après avoir atteint 25% de saturation en oxygène, est associée à une hyperglycémie de 60 minutes. Par la suite, le flux de glucose s'est abaissé aux valeurs normoxiques suggérant que l'utilisation *in situ* des réserves de glycogène tissulaire est une source de carburant plus importante pour la glycolyse anaérobie que le glucose circulant. L'hypoxie a aussi eu pour effet de diminuer de 52% le flux de NEFA indiquant que ce carburant n'est pas un substrat important au cours de l'hypoxie. Pendant un abaissement rapide de température (15°C à 6°C), les flux de glucose et de NEFA ont diminué proportionnellement à la dépression métabolique, conduisant à une valeur de Q_{10} d'approximativement 2. Il ne semble donc pas y avoir de préférence au niveau des substrats métaboliques utilisés au cours d'une exposition au froid. De plus, la concentration de glucose n'a diminué que très légèrement tandis que la concentration de NEFA a augmenté de 30% après 100 minutes à 6°C.

Les résultats des expériences sur l'hypoxie et sur l'abaissement de la température suggèrent que les changements de concentrations plasmatiques n'indiquent rien au sujet des modifications de flux. Cette thèse démontre que l'interprétation des changements de flux peuvent porter à confusion, et que la mesure directe des flux est nécessaire pour

comprendre la dynamique des substrats métaboliques au cours de manipulations expérimentales. La double cannulation et la technique d'infusion continue présentées dans cette thèse sont des outils nécessaires à l'étude des flux métaboliques *in vivo* chez les poissons. L'utilisation de cette technique peut maintenant être étendue à d'autres métabolites en états stables et instables tels la nage, les stress thermiques et osmotiques, et les traitements hormonaux.

REMERCIEMENTS

J'aimerais premièrement remercier mon superviseur Jean-Michel Weber pour m'avoir acceuilli dans son laboratoire et fait bénéficier de son expertise. Ses conseils et sa motivation ont rendu mon expérience d'étudiant gradué des plus enrichissantes. Les suggestions et les encouragements des membres de mon comité de thèse, Steve Perry et Frank Reardon, ainsi que de Thomas Moon ont aussi grandement bénéficié au déroulement de ma recherche.

Je souhaiterais aussi remercier ceux dont l'aide fut un atout majeur à la réussite de mon projet. Georges Zwingelstein m'a initié au merveilleux monde de la biochimie des lipides, et de part son expérience et son enthousiasme, m'a fait voir le monde de la physiologie comparée sous un nouvel oeil. Mark Powell et Nicholas Bernier ont été des collègues et des amis de première classe. Les longues discussions et les nombreuses expériences que nous avons partagées me resteront longtemps gravées dans la mémoire. Merci aussi à Katie Gilmour et Colin Brauner qui m'ont guidé patiemment à travers mes premières chirurgies.

Rien n'aurait été pareil sans mes fabuleux camarades de travail qui m'ont tous aider à leur façon: Servane Bernard, Shannon Reidy, Nancy Leblanc et Cassandra Grant (les femmes du laboratoire qui ont su m'endurer de peine et de misère), Gary Sullivan, Marosh Furimsky, Anna-Maria Greco, Lars Jürgensen, Wendy MacKenzie, Pascale Bellier, Manon Thibault, Tim Cano, Steve Dugan et Diana Otto. De plus, merci à

Stéphane Legault, Alain Brunet, Randa Dirani et Martin Boulerice des amis de longue date qui ont su m'encourager tout au long de ma recherche. Et surtout, merci à Josée Charron et à Darwin qui ont été à mes côtés dans les bons comme dans les moins bons moments. Finalement, je désirerais remercier mes parents Lyette et Germain Haman pour m'avoir encouragés à continuer mes études et permis d'emprunter un ordinateur sur lequel j'ai écrit une bonne partie de cette thèse.

TABLE OF CONTENTS

SUMMARY/RESUME.. iv

REMERCIEMENTS.. ix

LIST OF FIGURES.. xv

CHAPTER 1 - GENERAL INTRODUCTION.. 1

Glucose Fluxes.. 3

Lipid Fluxes.. 5

Some Environmental Perturbations that Can Influence Metabolite Fluxes.......... 10

Hypoxia.. 10

Temperature.. 12

Goals of the Investigation.. 13

CHAPTER 2 - A NEW CONTINUOUS TRACER INFUSION METHOD TO MEASURE IN VIVO METABOLITE TURNOVER RATES IN TROUT............ 15

Introduction.. 16

Materials and Methods.. 18

Experimental Animals.. 18

Placement of Catheters.. 18

Measurement of Glucose Turnover Rate.. 22

Metabolic Rate Measurement.. 23

Results.......... 25

Catheterization.......... 25

Continuous Isotope Infusion.......... 25

Glucose Kinetics.......... 27

Discussion.......... 30

CHAPTER 3 - RELIABILITY OF THE CONTINUOUS INFUSION METHOD TO MEASURE GLUCOSE TURNOVER RATE IN RAINBOW TROUT.......... 36

Introduction.......... 37

Materials and Methods.......... 39

Experimental Animals.......... 39

Surgical Procedure.......... 39

Experimental Protocol.......... 39

Sample Analysis.......... 41

Statistics.......... 41

Results.......... 42

Discussion.......... 47

CHAPTER 4 - EFFECTS OF ACUTE HYPOXIA AND COLD EXPOSURE ON NEFA AND GLUCOSE KINETICS 52

Introduction 53

Hypoxia 53

Temperature 58

Materials and Methods 60

Experimental Animals 60

Surgical Procedure 60

Choice of Radioactive Tracer 60

Preparation of the Infusate and Continuous Isotope Infusion 61

Oxygen Consumption Measurements 63

Experimental Protocol 63

Sample Analysis 64

Calculations and Statistics 69

Results 73

Plasma NEFA Concentrations and % Composition 73

Distribution of ^{14}C Activity in Plasma Lipids 73

Hypoxia Experiments 77

Cold Exposure Experiments 82

Discussion 100

Effect of Hypoxia 100

Effect of Cold Exposure 103

Effect of Hypoxia and Cold Exposure on Plasma NEFA Composition. 105

CHAPTER 5 - GENERAL CONCLUSION.. 108

REFERENCES.. 113

LIST OF FIGURES

Figure 1.1 - Carbohydrate pathway........ 7

Figure 1.2 - Lipid pathway........ 9

Figure 2.1 - Double dorsal aorta cannulation........ 19

Figure 2.2 - Resting glucose turnover rate measured using a continuous infusion........ 29

Figure 2.3 - Resting glucose turnover rates measured in resting teleosts........ 34

Figure 3.1 - Glucose turnover rate during validation experiments........ 46

Figure 4.1 - Sample analysis - Glucose and NEFA concentrations and activities........ 67

Figure 4.2 - Plasma lipid repartition following thin layer chromatography........ 71

Figure 4.3 - Effect of hypoxia on oxygen consumption........ 79

Figure 4.4 - Effect of hypoxia on glucose kinetics........ 81

Figure 4.5 - Continuous infusion of 1-^{14}C-palmitate during hypoxia........ 84

Figure 4.6 - Effect of hypoxia on NEFA kinetics........ 86

Figure 4.7 - Effect of cold exposure on oxygen consumption........ 88

Figure 4.8 - Effect of cold exposure on glucose kinetics........ 91

Figure 4.9 - Continuous infusion of 1-^{14}C-palmitate during cold exposure........ 93

Figure 4.10 - Effect of cold exposure on NEFA kinetics........ 95

Figure 4.11 - Glucose, NEFA and oxygen fluxes during cold exposure........ 98

CHAPTER 1

GENERAL INTRODUCTION

Metabolic fuels, end-products, and anabolic precursors are constantly produced and used and they must be transported between tissues via the cardiovascular system at appropriate rates and times. The regulation of metabolite fluxes is therefore essential in maintaining homeostasis even under severe changes in environmental conditions. To coordinate biochemical processes involved in maintenance, growth, reproduction, locomotion and various responses to environmental stresses, fish must constantly adjust rates of metabolite turnover (Weber and Zwingelstein, 1995). In studies of fish metabolism, blood concentration of key metabolites have been monitored to examine the biochemical consequences of various stresses. In this context, turnover rate is a much more informative parameter than concentration, and the temptation to use concentration changes to draw conclusions about fluxes has not always been resisted. Such extrapolation is clearly unwarranted, however, because flux and concentration do not necessarily change in parallel (Wolfe, 1992).

Reliable methods to measure metabolite turnover rates *in vivo* are widely available (Hetenyi *et al.*, 1983), and they have been used extensively in mammalian metabolic studies, especially for humans (Wolfe, 1992). In comparison, surprisingly little work has been carried out on fish (Garin *et al.*, 1987; Weber and Zwingelstein, 1995), mainly because it has been difficult to adapt tracer techniques to aquatic animals of smaller relative body size. The *bolus injection* method has been used almost exclusively in fish studies (Weber and Haman, 1996) because it only requires a single catheter, and can consequently be performed with a simple dorsal aorta cannulation. This method has serious limitations, however, and it has been almost totally abandoned for mammals

because it can only be used under steady-state conditions, and each experiment allows the calculation of a single turnover rate after analysis of a minimum of 6 to 10 blood samples. In mammals, metabolite turnover rates are quantified with the more versatile *continuous infusion* technique because it allows measurements under steady as well as non-steady state conditions; also, much more information can be obtained from a single experiment because instantaneous flux can be calculated from each blood sample separately. Unfortunately, this method requires the surgical placement of two catheters - one to infuse the metabolic tracer, the other for blood sampling - and it has never been adapted to investigate the metabolism of rainbow trout (from now on when I mention trout it refers to rainbow trout, *Oncorhynchus mykiss*), the most commonly used teleost model.

GLUCOSE FLUXES

Carbohydrates are only thought to play a minor role as a metabolic fuel in fish. The long term use of this fuel at high rates is probably not compatible with its very low availability in their diets (Halver, 1972). It is surprising to notice that almost no quantitative information on rates of appearance and disappearance of blood glucose is available for fish *in vivo*. However, one recent study stands out as an exception: West *et al.* (1993) adapted the deoxyglucose method developed by Sokoloff for cerebral metabolism (1983) to quantify rates of local glucose utilization by swimming fish red muscle and heart; they found that glucose accounts for less than 10% of the metabolic rate of these tissues. All other published glucose flux measurements have been carried out in resting fish, and their validity is often questionable. In all cases, radiolabeled glucose has

been administered, either as a bolus injection (Andersen *et al.*, 1991; Bever *et al.*, 1977; Bever *et al.*, 1981; Dunn and Hochachka, 1987; Garin, *et al.* 1987; Lin *et al.*, 1978; Machado *et al.*, 1989; Vijayan and Moon, 1994; Weber *et al.*, 1986), or, in one instance, as a continuous infusion (Cornish and Moon, 1985). The bolus injection technique requires rapid mixing of the label within the total glucose pool, before taking immediate and frequent measurements of glucose specific activity within the same pool (Wolfe, 1992). Rapid mixing was clearly not achieved in studies where labeled glucose was injected intraperitoneally (Garin *et al.*, 1987; Lin *et al.*, 1978), and, in other investigations, the first, and crucial part of the specific activity decay curve was missed (Andersen *et al.*, 1991; Machado *et al.*, 1989), making it very difficult to quantify turnover adequately. Also, the assumption of steady glucose concentration associated with this technique has not always been satisfied because fish tend to become hyperglycemic (Andersen *et al.*, 1991; Bever *et al.*, 1977; Dunn and Hochachka, 1987; Vijayan and Moon, 1994), or sometimes even hypoglycemic (Garin *et al.*, 1987) when stressed by repeated blood sampling. In some studies, measurements were made in semi-anesthetized animals, a treatment that probably depresses carbohydrate metabolism (Machado *et al.*, 1989; Weber *et al.*, 1986). The bolus injection technique has never been validated in fish. Finally, only American eels have been investigated by continuous infusion (Cornish and Moon, 1985), a more accurate and versatile technique allowing measurements under non-steady state conditions (Wolfe, 1992). Surprisingly, the turnover rate measured in eels was 10 to 90 times higher than for any other teleost except tuna (Weber and Zwingelstein, 1995). It is

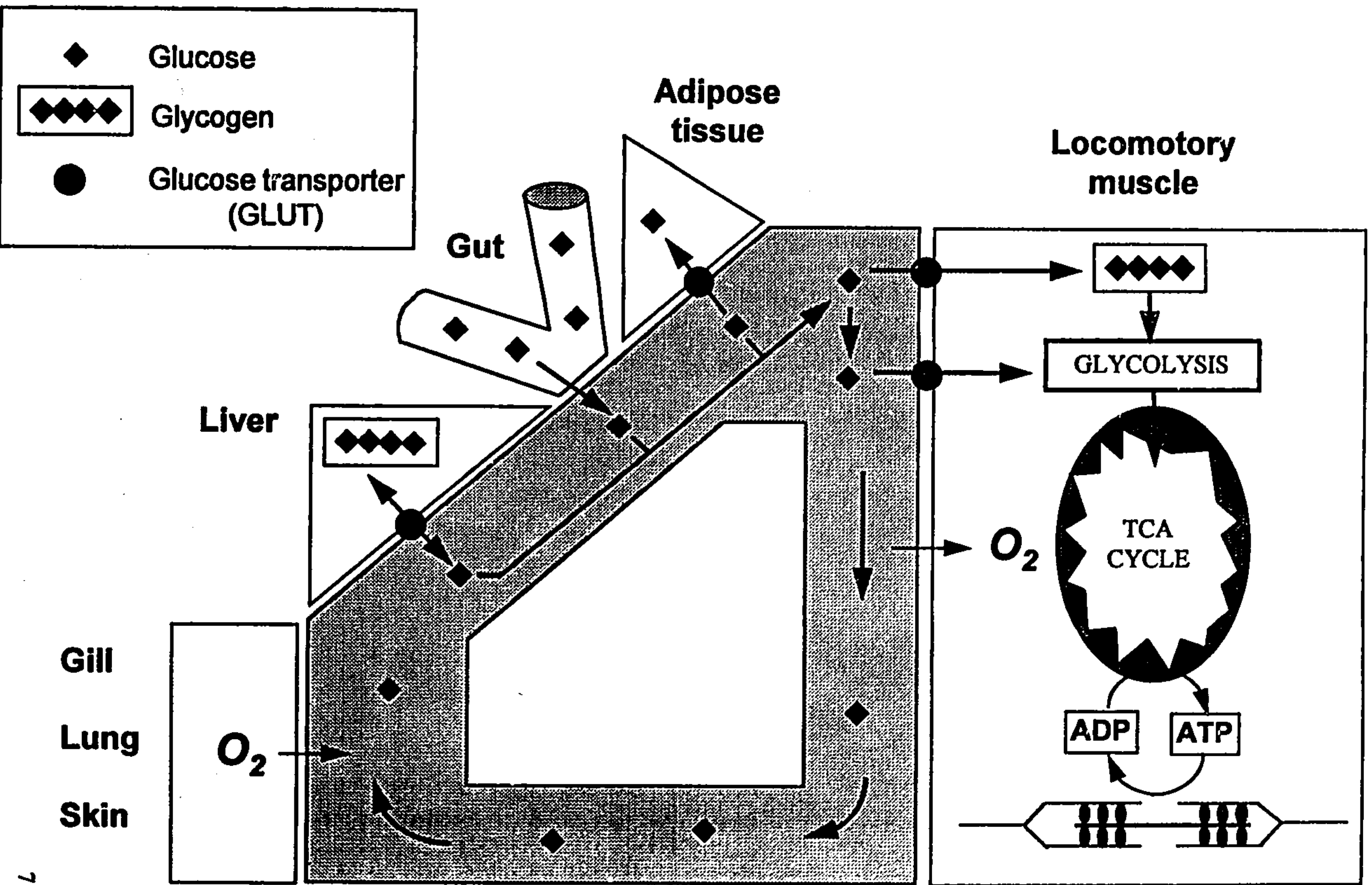
not clear whether this large discrepancy is due to true species differences or to the methodological problems cited above.

Figure 1.1 gives a general summary of the pathways for carbohydrate supply to tissue mitochondria. In fish, as in other vertebrates, glucose energy can be provided from the gut, the liver (glycogen stores or gluconeogenesis), and from intramuscular glycogen reserves. In mammals, glucose entry in all cells is mediated by tissue-specific transporters (GLUT) (Carruthers, 1990). The presence of such transporters in fish cell membranes has not been demonstrated, but there is no reason to believe that fish lack this carrier-mediated transport system.

LIPID FLUXES

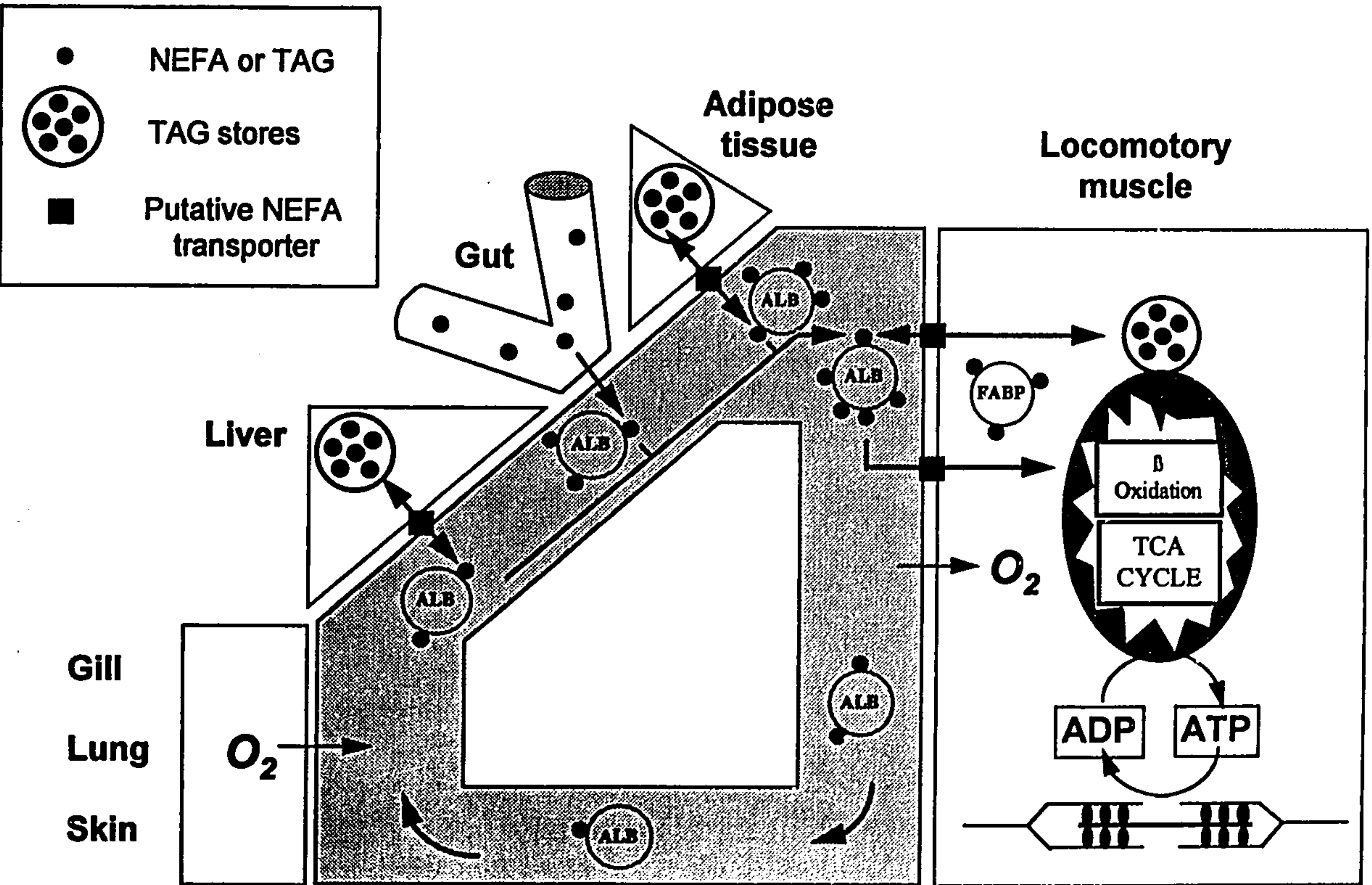
Unlike carbohydrates, lipids represent a significant component of most fish diets, and they are stored in relatively large quantities. Their oxidation accounts for an important fraction of energy metabolism in most tissues. The major pathways for lipid provision to muscle mitochondria are outlined in Figure 1.2. Only non-esterified fatty acids (NEFA) and triacylglycerol (TAG) are indicated as representative examples. NEFAs represent the most dynamic form of lipid that is transported from lipidic reserves to utilizing tissues (Sheridan, 1988). However, it is important to keep in mind that other classes of lipids, such as lipoproteins (Very Low Density Lipoproteins, Low Density Lipoproteins and High Density Lipoproteins), may also play a significant role in supplying energy for muscle contraction in fish. A more detailed account of their metabolism can be found in reviews by Sheridan (1988), and Weber and Zwingelstein (1995).

Figure 1.1 Pathway for carbohydrate supply to locomotory muscles. Simple carbohydrate subunits are derived from intestinal absorption or hepatic gluconeogenesis. They can be stored as liver glycogen, muscle glycogen, or converted to triacylglycerol in adipose tissue. Glucose exchange between liver, adipose tissue, blood and muscle is controlled by tissue-specific glucose transporters (GLUT) in cell membranes. (From Weber and Haman, 1996)



Glucose
Glycogen
Glucose transporter (GLUT)
Adipose tissue
Gut
Liver
Locomotory muscle
GLYCOLYSIS
TCA CYCLE
ADP
ATP
O_2
Gill
Lung
Skin
O_2

Figure 1.2 Pathway for lipid supply to locomotory muscles. Non-esterified fatty acids (NEFA) and triacylglycerol (TAG) of intestinal origin can be stored as liver, adipose tissue or muscle TAG reserves. Lipids are transported through plasma bound to an albumin-like protein, and through the cytosol bound to fatty acid binding proteins (FABP). NEFA exchange between liver, adipose tissue, plasma and muscle may be mediated in part by presumed NEFA transporters in cell membranes. (From Weber and Haman, 1996)


NEFA or TAG
TAG stores
Putative NEFA transporter
Gut
Adipose tissue
Locomotory muscle
Liver
ALB
FABP
β Oxidation
TCA CYCLE
O_2
Gill
Lung
Skin
O_2
ADP
ATP

In general, lipid sources for energy metabolism can come from the *circulation* or from *intramuscular reserves.* Circulatory lipids are transported from the gut, and from hepatic or adipose tissue stores to tissue mitochondria. To allow significant circulatory ransport, these lipids must be bound to a plasma protein similar to (Davidson *et al.*, 1989; Fellows and Hird, 1981; Maillou and Nimmo, 1993), or sometimes very different from mammalian albumin (Peters Jr and Davidson, 1991), thereby increasing their solubility in plasma. Similarly, lipid transport in the aqueous cytosol is mediated by smaller cell-specific fatty acid binding proteins (FABP) (Clarke and Armstrong, 1989; Sweetser *et al.*, 1987), but limited information is yet available concerning the presence of these proteins in fish cells (Stewart and Driedzic, 1988). Practically no *in vivo* measurements of lipid fluxes have been attempted in fish (Weber and Zwingelstein, 1995). This lack of information is probably due to the large variety of plasma lipids available for energy transfer and to the relative complexity of lipid chemistry compared with carbohydrates.

SOME ENVIRONMENTAL PERTURBATIONS THAT CAN INFLUENCE METABOLITE FLUXES

Hypoxia

Since oxygen solubility is rather low in water, many factors (e.g. high temperatures, excessive algal growth) can cause aquatic organisms to experience hypoxic conditions (van den Thillart, 1982; Van Ginneken *et al.*, 1995; van Raaij, 1994). Adjustments to their cardiovascular and respiratory systems allow most teleosts to

improve oxygen extraction and delivery to tissues over a wide range of oxygen levels in the water (for review see Perry and Wood, 1989). However, when a critical oxygen tension is reached, oxidative metabolism is insufficient to produce all the ATP needed by the organism and an activation of anaerobic pathways occurs (Van Ginneken *et al.*, 1995; van Raaij, 1994). Metabolic reorganization switches fuel preference from an essentially aerobic source under normoxia (i.e., lipids and proteins) to a more anaerobic substrate during hypoxia (i.e., carbohydrates)(van den Thillart, 1982; van Raaij, 1994). Strategies for coping with hypoxia occur not only in the way ATP is produced but also in the way it is utilized (van den Thillart, 1982). Hypoxia tolerant species have greater glycogen reserves in their tissues, use more efficient fermentation pathways and depress metabolism to maintain a close coupling between ATP production and utilization (reviewed in detail in Hochachka and Somero, 1984; van den Thillart *et al.*, 1982; Hochachka and Guppy, 1987). This allows them to survive periods of severe hypoxia and even anoxia (van Raaij, 1994). However, these strategies to tolerate conditions of low oxygen availability are not employed by all teleosts. The response of salmonids, including rainbow trout, have been extensively studied under hypoxic conditions (e.g., Boutilier *et al.*, 1988; van Raaij, 1994; Dunn and Hochachka, 1986; 1987). These studies have shown that trout are among the most sensitive fish to oxygen deprivation. Hence, acute hypoxia effects on circulatory glucose and nonesterified fatty acids (NEFA) in rainbow trout may be much more apparent than in a hypoxia tolerant species.

Temperature

In living organisms, temperature influences physiological processes in many ways, but among the most important are its effects on ionization, protein structure and reaction rates (for review see Hochachka and Somero 1984, Clarke, 1993). Endotherms and ectotherms have widely different strategies for coping with changes in environmental temperature. Endotherms succeed well in defending their body temperature within a narrow range through various strategies. Biochemical and physiological processes can therefore be maintained at stable levels independently of changes in environmental temperature (Hochachka and Somero, 1984). On the other hand, ectotherms have virtually no defense mechanisms to maintain body temperature with changes in environmental temperature. Since variation in environmental temperature can be modified on various time scales and amplitudes (Clarke, 1993), the most ubiquitous restructuring of biochemical systems to offset (or exploit) the effects of temperature can be found in this group of animals (Hochachka and Somero, 1984). In aquatic ectotherms a temperature gradient between ambient water and body temperature is absent because of the high thermal capacity of water and efficiency of the gills to exchange heat (Clarke, 1993; Hazel and Prosser, 1974). Physiological and biochemical problems associated with acute cold exposure in fish include perturbations of biomembrane fluidity (for review see Hazel, 1984; Hendersen, 1987), changes in catalytic rate of enzymes, (for review see Hochachka and Somero, 1984; Sidell and Hazel, 1984, 1987; Wojcieszyz *et al.* 1981; Guderley, 1990), and diffusive processes of metabolites and respiratory gases within the tissues

(Sidell and Hazel, 1987). These temperature-dependent modifications compromise cell integrity and function (Hendersen, 1987). While compensatory adjustments to minimize these effects of temperature during acclimation have been extensively studied in fish, very little information is known on the effects of acute temperature change on the regulation of circulatory metabolic fuels.

GOALS OF THE INVESTIGATION

The general aim of my thesis is to measure changes in glucose and nonesterified fatty acid fluxes in rainbow trout acutely exposed to hypoxia or cold. The work is divided in three parts:

1. Development of a surgical technique for the placement of 2 catheters to measure *in vivo* metabolite fluxes by continuous infusion in trout (Chapter 2)

2. Determine how accurately glucose flux can be estimated with this new continuous infusion method (Chapter 3)

3. Measure the effects of two environmental stresses, hypoxia and cold exposure, on glucose and NEFA fluxes (Chapter 4).

In more detail, Chapter 2 describes a new double dorsal aorta catheterization technique allowing the measurement of substrate turnover rate by continuous infusion of

metabolic tracers in rainbow trout. As a practical example of a routine substrate flux measurement, glucose turnover rate of resting trout is measured by primed continuous infusion of 6-^{3}H-glucose through one of the catheters, and blood sampling from the other. Chapter 3 describes the validation of the continuous infusion method developed in Chapter 2. The validation technique used by Allsop *et al.* (1978) to verify the reliability of glucose flux measurement in mammals is applied to trout. While anesthetized, trout are hepatectomized and liver glucose production is replaced by a pump infusing unlabelled glucose at a known rate. Glucose turnover rates are assessed using either plasma or whole blood as described in Chapter 2. In Chapter 4, the continuous infusion technique developed in Chapter 2 is used to study the effect of acute hypoxia and cold exposure on circulatory glucose and NEFA. During hypoxia experiments, water oxygen saturation is decreased in 20 minutes from 100% (normoxia) to 25% (hypoxia). The animals are then maintained under hypoxic conditions for a period of 90 minutes. Low oxygen availability causes a switch in fuel utilization from essentially aerobic substrates (i.e., proteins and lipids) to a more anaerobic one (carbohydrates). Therefore, my hypothesis is that trout exposed to hypoxia will increase their glucose flux and decrease their NEFA flux. During the cold exposure experiments, 15°C-acclimated trout are exposed to a 20 minute decrease (10°C) in water temperature. My hypothesis is that both glucose and NEFA fluxes will decrease in proportion to metabolic rate depression.

Finally, general conclusions are presented in Chapter 5 where the common use of changes in plasma metabolite concentrations to interpret the effects on environmental stresses is questioned.

CHAPTER 2

A NEW CONTINUOUS TRACER INFUSION METHOD TO MEASURE *IN VIVO* METABOLITE TURNOVER RATES IN TROUT

INTRODUCTION

The most versatile technique to measure metabolite flux *in vivo* - the continuous infusion method - has never been used in trout. This is probably due to the surgical difficulties encountered when placing two catheters needed for simultaneous isotope infusion and blood sampling. With the exception of one study in eels (*Anguilla rostrata*) (Cornish and Moon, 1985), bolus injection has always been used to estimate glucose turnover rate in teleosts (see Figure 3 for references), probably because single cannulations were sufficient for this method. Unfortunately, the bolus injection technique can only provide one flux measurement per experiment, and the animal has to be in steady-state. Therefore, the need for an adequate double cannulation technique to measure glucose flux by continuous infusion becomes apparent.

The now classic dorsal aorta cannulation technique of Smith and Bell (1964), later modified by Soivio *et al.* (1975), revolutionized the study of fish metabolism by allowing repeated blood sampling and intravascular injections in undisturbed, non-anaesthetized fish. Over the last 30 years, dorsal aorta cannulation has been used routinely to investigate fundamental aspects of fish biochemistry and physiology. This surgical approach has provided invaluable information on the effects of various stresses including exercise, changes in water temperature, pH, salinity, oxygen content, and toxicant concentration. In studies of fish metabolism, blood concentrations of key metabolites have been monitored to examine the biochemical consequences of these stresses. In this context, turnover rate is a much more informative parameter than concentration. Moreover, flux

and concentration do not necessarily change in parallel (Wolfe, 1992). A very large change in turnover rate can be associated with an increase, no change, or even a decrease in concentration, depending on the prevailing difference between rates of appearance and disappearance. Therefore, my goals here were to develop an easy surgical technique to perform double catheterization of rainbow trout, and to use the continuous tracer infusion technique to quantify resting glucose turnover.

MATERIALS AND METHODS

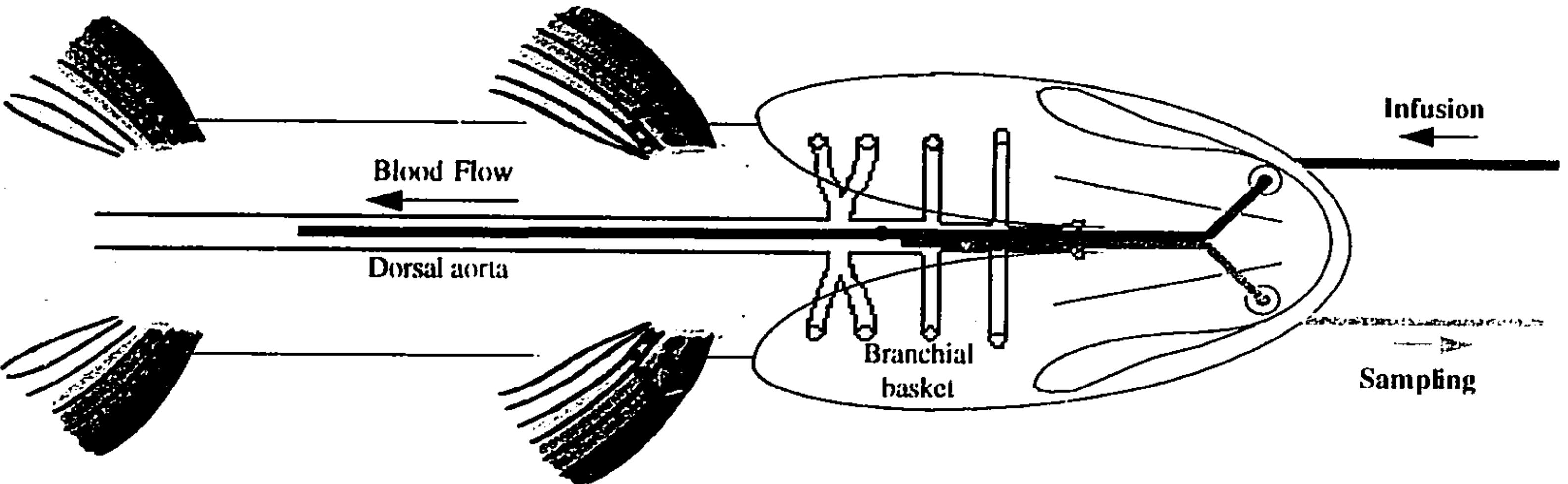
Animals

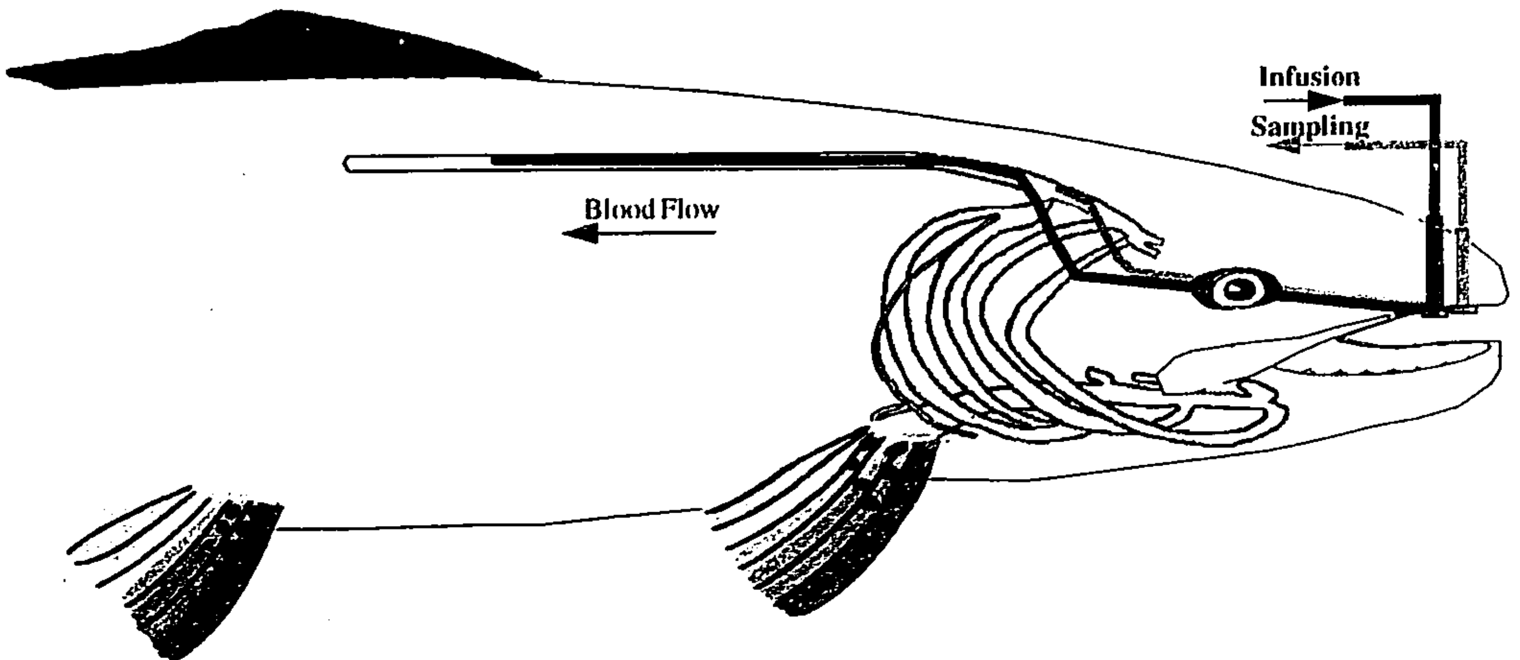
Rainbow trout of both sexes (831 to 1265 g) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario), and held in 550-liter flow-through tanks at 15 °C. They were kept in dechloraminated, well oxygenated Ottawa tap water under 12 h:12 h light:dark photoperiod. The animals were acclimated to these conditions for at least 1 month before running experiments and they were fed Purina trout chow 3 times a week until satiation.

Placement of catheters

Twenty-four hours before isotope infusion, each animal was anesthetized in 0.1 g/l ethyl-N-aminobenzoate sulphonic acid (MS-222) buffered with 0.2 g/l bicarbonate. During surgery, the anaesthetic solution was recirculated and aerated to perfuse the gills. The cannulation technique of Soivio *et al.* (1975) was modified to allow the placement of two separate PE-50 catheters (Intramedic, Clay-Adams) into the dorsal aorta. The first catheter was used for isotope infusion and it was inserted at the 3rd gill arch before being fed 8-10 cm caudally into the vessel. The second catheter was used for blood sampling. It was entered in the artery at the 1st gill arch and was fed caudally no more than 2 cm (see Figure 2.1 a, b). These infusion and sampling sites provide an accurate measurement of glucose turnover because the determination of glucose kinetics does not depend on exact catheter placement (Katz, 1992; Norwich, 1992; Wolfe, 1992). Each catheter was filled with heparinized (10 U/ml) Cortland saline without glucose (Wolf, 1963), and held

Figure 2.1. Anatomical diagram indicating the location of isotope infusion (purple) and sampling catheters (orange) in the dorsal aorta of rainbow trout. The PE-50 catheters are drawn larger than scale to emphasize their exact position. a) ventral view - dots (●) represent catheter entry sites in the artery b) lateral view.


Infusion
Blood Flow
Dorsal aorta
Branchial
basket
Sampling


Infusion
Sampling
Blood Flow

securely in place by 2 stitches to the roof of the mouth. After recovery from anaesthesia each animal was placed in an opaque Plexiglas chamber (60 x 16 x 18 cm) supplied with the same quality water as the holding tanks at 5-6 l/min. The chamber was sealed with a lid tightly held in place with screws and both catheters were allowed to exit the chamber through a small opening in the lid. All experiments were carried out between 24 and 48 h after surgery.

Measurement of glucose turnover rate

While the fish was resting quietly in the Plexiglas chamber, a continuous infusion of 6-^{3}H-glucose (New England Nuclear 1.6 TBq/mmol or Amersham 1.11 TBq/mmol) was started with a calibrated syringe pump at 1 ml/h (Harvard Apparatus, South Natick, MA). Glucose tritiated in position 6 has been shown to yield the best estimate of glucose turnover because detritiation due to hepatic cycling is minimal (Katz, 1979). A priming dose equivalent to 90 min of infusion was injected as a bolus before starting the pump to decrease the time necessary to reach isotopic steady state in the relatively large glucose pool (Wolfe, 1992). The infusate was prepared daily by drying an aliquot of the stock solution under N_2 and redissolving it in Cortland saline without heparin. Exact infusion rate was determined for each experiment by counting an aliquot of the infusate; infusion rates ranged between 30 059 and 108 333 DPM kg^{-1} min^{-1}. In the results section, glucose specific activities have been divided by infusion rate for each experiment to provide standardized values allowing meaningful comparisons. Blood samples (300 µl each) were drawn after 40, 50, and 60 min of infusion. Each sample was diluted in 500 µl perchloric

acid (8%) and centrifuged. After separation, the perchloric acid extracts were used to measure glucose and lactate concentrations at 340 nm via standard enzymatic methods (Bergmeyer, 1985) on a Milton Roy spectrophotometer (Spectronic 1001). Glucose specific activity was measured by neutralizing perchloric acid extracts in 3 M K_2CO_3 in 0.5M triethanolamine hydrochloride. An aliquot of 60 µl was then placed to dry in a 20 ml scintillation vial under a stream of N_2 gas before being resuspended in 1ml H_2O (d) and 10 ml ACS II scintillation fluid (Amersham, Oakville Ontario). This procedure was used because total tritium activity has been shown to be restricted to glucose and water (Katz *et al.*, 1974). Therefore, this simple technique to determine specific activity is routinely used for glucose turnover measurements in dogs (Moates *et al.*, 1988), humans (Levy *et al.*, 1989; Molina *et al.*, 1990), birds (Marsh and Dawson, 1982), and fish (West *et al.*, 1994a; West *et al.*, 1994b). Counting was performed on a Tri-Carb 2500 scintillation counter (Packard, Canada) with external quench correction. Glucose turnover rate (R_t) was calculated with the steady-state equation of Steele (1959)

$$R_t = \frac{F}{SA}$$

where F and SA represent isotope infusion rate and metabolite blood specific activity (SA= isotope activity / metabolite blood concentration), respectively. Glucose clearance rate (MCR) was determined by dividing turnover rate by glucose concentration.

Metabolic rate measurement

Oxygen consumption was measured by stopping external water supply 3 times for a 10 min period during each isotope infusion, and recycling the chamber water within a 15

liter closed system. Particular care was taken to eliminate air bubbles within the system and to avoid exchange between the recirculated water and atmospheric air. Water flow rate through the chamber remained at 5-6 l/min during the metabolic rate measurements. During each 10 min interval, O_2 concentration was recorded every min with a calibrated oxygen electrode (Oxyguard, Handy MK III, Valox ltd). The total decrease in PO_2 never exceeded 10% of saturation PO_2 but the change was large enough to measure oxygen consumption accurately. The total volume of recirculated water (15 l) was selected to provide an optimal ratio of fish size to respirometer volume (Steffensen, 1989). The rate of oxygen consumption (MO_2) was calculated with the closed system equation of Steffensen (1989). After each metabolic rate measurement, return to saturation PO_2 was accomplished by flushing the system for 5 min. All results are given as means ± S.E.M.

RESULTS

Catheterization

The double cannulation technique proposed in this paper is summarized in Figure 2.1 where exact catheter placement is indicated. Complete surgery was achieved in 25–40 min depending on the individual fish (surgery time = time elapsed between starting the anaesthesia and complete recovery with the animal having regained its normal balance). Surgical success rate was higher than 90% with the large rainbow trout used here (see Table 2.1). Additional experiments (results not shown) revealed that it is possible to cannulate smaller animals down to 600 g with the same success rate, however, performing this double catheterization in animals smaller than 500 g became increasingly difficult because of the lack of working space within the mouth of the animal. The surgical procedure caused minimal blood loss as indicated by the maintenance of high hematocrits (Table 2.1). After removing catheterized fish from the anaesthesia solution, recovery occurred in 5-10 min.

Continuous isotope infusion

Both catheters were kept patent between surgery and isotope infusion without problems. The opaque Plexiglas chamber, low infusion rate (1 ml/h) and good sampling catheters allowed isotope infusion and blood sampling to occur without disturbing the fish, as indicated by the normal resting metabolic rates measured during the experiments (MO_2 ranged between 33.5 and 63.3 μmol O_2 kg^{-1} min^{-1}, and it averaged 42.2 ± 3.7, see Table 2.1). In addition, no thrashing or unusual movements were observed as a result of isotope

Table 2.1. Metabolic rate (MO_2), and blood glucose concentration, turnover rate (R_t), and clearance rate (MCR) in resting, post-absorptive rainbow trout during continuous infusions of 6-^{3}H-Glucose at 15°C. Body mass (M_b), hematocrit (Hct) and blood lactate concentration are also indicated. Within each experiments values given are means ± SE M (n=3 blood samples at 40, 50 and 60 min of infusion).

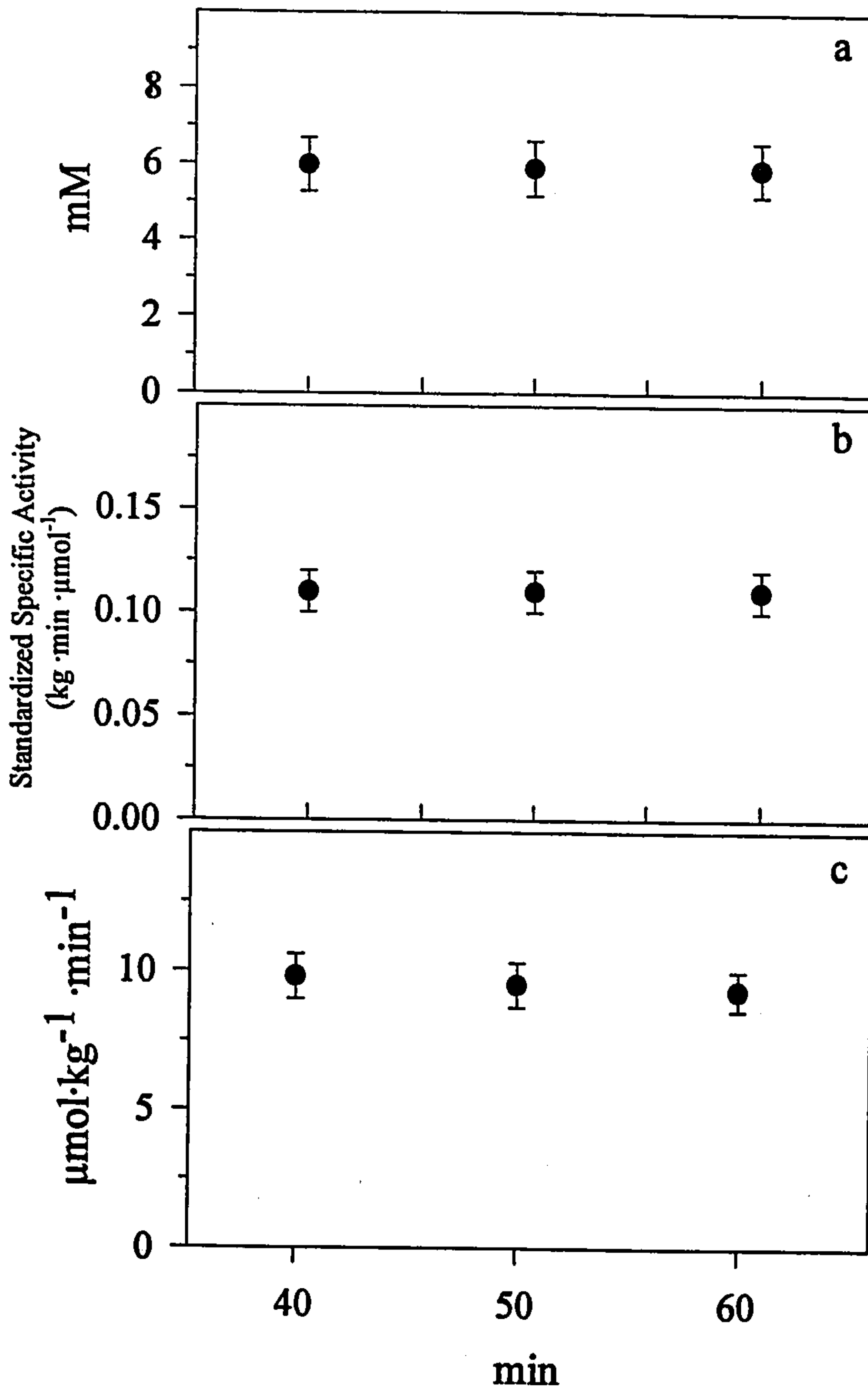
Expt.#	M_b (g)	Hct (%)	Lactate (mM)	MO_2 ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)	Glucose (mM)	R_t ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)	MCR ($ml \cdot kg^{-1} \cdot min^{-1}$)
1	1036	28	0.6 ± 0.11	40.0 ± 2.4	8.7 ± 0.1	11.7 ± 0.4	1.3
2	831	18	0.6 ± 0.02	40.4 ± 0.5	6.6 ± 0.1	10.5 ± 0.0	1.6
3	1265	25	0.6 ± 0.02	63.3 ± 2.7	3.6 ± 0.1	7.1 ± 0.3	2.0
4	831	21	0.6 ± 0.02	33.5 ± 0.8	6.3 ± 0.1	10.0 ± 0.2	1.6
5	915	32	1.6 ± 0.00	43.3 ± 1.0	3.9 ± 0.1	11.0 ± 0.1	2.8
6	1168	30	0.7 ± 0.07	35.1 ± 2.1	7.9 ± 0.1	6.9 ± 0.1	0.9
7	1011	36	2.3 ± 0.06	--------	3.4 ± 0.1	7.1 ± 0.3	2.1
8	913	33	1.3 ± 0.12	40.0 ± 2.3	6.8 ± 0.1	12.0 ± 0.3	1.8
Average	996	27.5	0.8	42.2	5.9	9.5	1.8
SEM (n=8)	55	2.2	0.2	3.7	0.7	0.8	0.2

infusion or blood sampling and low blood lactate and normal blood glucose concentrations were maintained throughout the infusion procedure (Table 2.1).

Glucose kinetics

The infusion rates, priming dose and blood sampling schedule selected here were adequate to achieve concentration and isotopic steady state conditions for glucose in 40 min of infusion. Glucose concentration, specific activity and turnover rate did not change significantly over time (Figure 2.2, $P>0.05$, ANOVA). Results for individual infusion experiments are presented in Table 2.1. The resting glucose turnover rate of rainbow trout ranged between 6.9 and 12.0 $\mu mol\ kg^{-1}\ min^{-1}$ and averaged 9.5 ± 0.8 $\mu mol\ kg^{-1}\ min^{-1}$. Turnover rate and concentration were not significantly correlated ($r=0.49$, $P = 0.22$, $n=24$). However, the power of the performed test (0.67) was below the desired power of 0.8 indicating that the number of animals may not have been sufficient to determine this correlation. Glucose clearance rate ranged between 0.9 and 2.8 $ml\ kg^{-1}\ min^{-1}$ and averaged 1.8 ± 0.2 $ml\ kg^{-1}\ min^{-1}$.

Figure 2.2. Blood glucose concentration (a), standardized glucose specific activity (= specific activity / infusion rate) (b), and glucose turnover rate (c) during primed continuous infusions of 6-^{3}H-glucose in rainbow trout. Values are means ± SEM (n=8).


a
8
6
4
2
0
mM
b
0.15
0.10
0.05
0.00
Standardized Specific Activity
(kg ·min ·µmol-1)
c
10
5
0
µmol·kg-1 ·min-1
40
50
60
min

DISCUSSION

This chapter describes a double dorsal aorta catheterization technique allowing the easy measurement of substrate turnover rates by continuous infusion of metabolic tracers in rainbow trout. The time necessary to carry out this new surgical procedure is only slightly longer than for a single aortic cannulation (Soivio *et al.*, 1975), and it can be performed with minimal training. Moreover, the animals recover rapidly from a simple operation causing negligible blood loss and no more stress than the commonly used single cannulation. Other, less satisfactory techniques were considered before selecting the method described here; the most promising alternative was to place the infusion catheter in the coeliac artery and to sample blood from the dorsal aorta. However, after several attempts this approach was eventually abandoned because coeliac artery cannulation is far more invasive, and therefore much more stressful (i.e. longer surgery time, more blood loss, and a large external wound to heal). In addition, isotopic steady state could not be reached when labeled glucose was administered through the coeliac artery, even after prolonged infusion.

The technique proposed here makes it possible to measure metabolic substrate fluxes by *continuous infusion* in rainbow trout, instead of relying on the commonly used, but much less versatile *bolus injection* method (Weber and Haman, 1996; Weber and Zwingelstein, 1995). There are 3 main reasons why using continuous infusion is advantageous for fish metabolism studies: 1) this technique has been shown to yield much more accurate estimates of substrate flux than bolus injection under steady state conditions

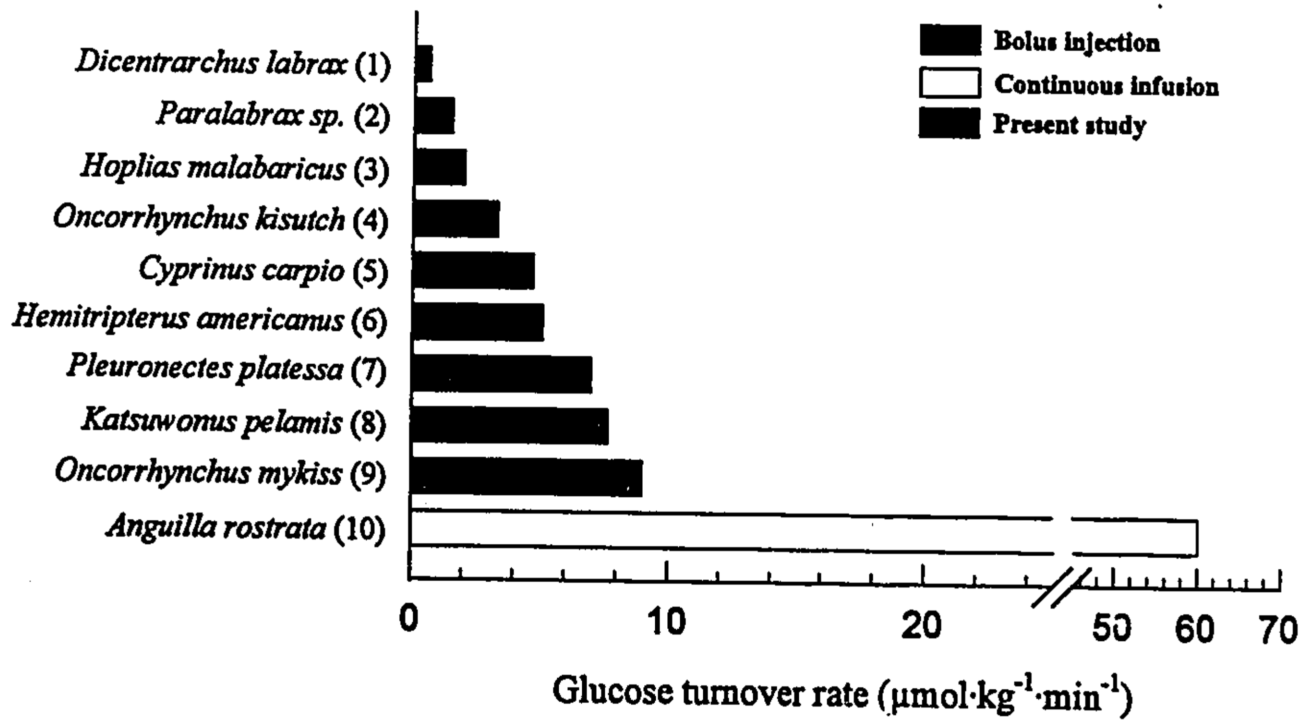
(i.e., when concentration and flux of the metabolite investigated are constant throughout the experiment) (Allsop *et al.*, 1979; Allsop *et al.*, 1978); 2) bolus injection cannot be used to monitor rates of appearance and disappearance independently under non-steady state conditions (Hetenyi *et al.*, 1983); and 3) a single value of flux can be measured with much less blood (i.e. only one or 2 blood samples are necessary by continuous infusion, whereas a complete specific activity decay curve must be generated with bolus injection) (Wolfe, 1992).

As a practical example, glucose kinetics of resting rainbow trout were measured to establish the necessary experimental conditions for future use of the proposed technique in this species. If the glucose pool is initially primed with the equivalent of a 90 min infusion, isotopic steady state is achieved in less than 40 min with infusion rates ranging between 30 000 and 110 000 DPM kg^{-1} min^{-1}. For the measurement of glucose turnover, injecting a priming dose is necessary in order to reach steady state rapidly, because the total glucose pool of vertebrates is known to be large (reaching steady state without priming the pool would take several hours). In all my experiments, glucose concentration and specific activity remained constant beyond 40 min (Figure 2.2), and these results show that, beyond this time, the proposed experimental set-up can be used to quantify rates of glucose production and disposal under non steady state conditions if required (e.g. during swimming, when water quality is altered, or when hormonal manipulations are imposed on the animal).

Resting glucose turnover rates of rainbow trout averaged 9.5 ± 0.8 µmol kg^{-1} min^{-1} (Table 2.1) and variability among individuals was low (R_t ranging between 6.9 and 12.0 µmol kg^{-1} min^{-1} (n=8)). In addition, all my fish were in a true resting state during the

infusions because they showed normal resting metabolic rates for this species, and their blood lactate concentration remained low throughout the experiments. I was able to find three other comparable published values of resting glucose R_t in rainbow trout, all of them measured by bolus injection at 13-15 °C (Andersen *et al.*, 1991; Cowey *et al.*, 1977; Washburn *et al.*, 1992). These values were 4-9 times lower (ranging from 1 to 2.4 µmol kg^{-1} min^{-1}) than what I measured here by continuous infusion. It is difficult to compare these values with mine because the bolus injection method is much more prone to experimental errors. In the studies by Andersen *et al.* (1991) and Cowey *et al.* (1977), the first blood sample was only taken 60 min after isotope injection, and the most crucial portion of the decay curve was therefore missed. An accurate estimate of the surface area under the specific activity decay curve would only have been possible if the early part of the curve had been defined adequately (i.e. many samples must be taken in the first 60 min after injection to generate a usable decay curve). Furthermore, their animals were not truly at rest; they were taken repeatedly out of water to inject the isotope and to sample blood from the caudal vein (Cowey *et al.*, 1977), they showed hyperglycemia (Andersen *et al.*, 1991), or they were anaesthetized with MS-222 shortly before isotope injection (Washburn *et al.*, 1992). Contrary to these 3 studies, West *et al.* (1994b) recently found resting glucose turnover rates twice as high as were measured here, but these high fluxes were probably caused by an unusually high plasma glucose concentration (averaging 20 mM). In the present study, glucose concentration and turnover rate were not correlated. In contrast, West *et al.* (1994a; 1994b) found a significant correlation in both,

Figure 2.3. Resting glucose turnover rates measured by bolus injection or continuous infusion in 10 teleost species. Measurements made at different temperatures have been corrected to 15°C using a Q_{10} of 2. References: (1) Garin *et al.*, 1987; (2) Bever *et al.*, 1977; 1981; (3) Machado *et al.*, 1989; (4) Lin *et al.*, 1978; (5) West *et al.*, 1994a; (6) Vijayan and Moon, 1994; (7) Batty and Wardle, 1979; (8) Weber *et al.*, 1986; (9) present study; (10) Cornish and Moon, 1985.



Bolus injection
Continuous infusion
Present study
Dicentrarchus labrax (1)
Paralabrax sp. (2)
Hoplias malabaricus (3)
Oncorrhynchus kisutch (4)
Cyprinus carpio (5)
Hemitripterus americanus (6)
Pleuronectes platessa (7)
Katsuwonus pelamis (8)
Oncorrhynchus mykiss (9)
Anguilla rostrata (10)
0
10
20
50
60
70
Glucose turnover rate (μmol·kg-1·min-1)

hyperglycemic trout (6-38 mM plasma glucose) and carp (3-17 mM). These results suggest that flux and concentration only show a significant correlation when the animals become hyperglycemic.

Resting glucose turnover rates were also measured by various researchers in several other fish species. Figure 2.3 summarizes the published results. Except for a single study where continuous infusions were carried out in American eels (Cornish and Moon, 1985), fish glucose turnover rates were always measured by the bolus injection technique. Interestingly, bolus injection experiments have all provided lower estimates of turnover rate than were observed here for trout, whereas the American eel values previously obtained by continuous infusion were 6 times higher. Critical problems with the use of bolus injection have been mentioned above, or discussed previously (Weber and Haman, 1996). Because continuous infusion bypasses many of these problems (Wolfe, 1992), and because it is known to be more accurate than bolus injection (Allsop *et al.*, 1979; 1978), previous estimates of glucose turnover measured by bolus injection probably underestimate true rates. In Chapter 3, the reliability of the continuous infusion technique will be tested for the measurement of glucose flux in rainbow trout.

CHAPTER 3

RELIABILITY OF THE CONTINUOUS INFUSION METHOD TO MEASURE GLUCOSE TURNOVER RATE IN RAINBOW TROUT

INTRODUCTION

In chapter 2, I developed a new cannulation technique to measure metabolite fluxes via continuous infusion, and showed that glucose flux values obtained with this method are much higher than the values obtained previously by bolus injection. In mammals, the continuous infusion technique was validated by Allsop *et al.*,. (1978). While maintaining the animal under anesthesia, their method consisted in surgically removing endogenous glucose sources, and in replacing them by an external pump infusing unlabelled glucose at a known rate. A similar experimental approach to this classic paper is used here for fish. Continuous infusion of labeled glucose is performed on hepatectomized fish to check whether the known rate of unlabelled glucose entry is measured accurately by the isotope dilution technique.

As in mammals, the liver is the main source of glucose in fish, and kidneys are only very minor contributors, mainly because renal activities of key gluconeogenic enzymes are extremely low in trout (Knox *et al*, 1980; Suarez and Mommsen, 1987). Here, kidneys were left intact because their removal from fish cannot be performed without causing major damage to the circulatory system and surrounding tissues.

Estimates of glucose flux are normally calculated from rates of isotope infusion and glucose specific activities in the circulation. In mammalian studies, specific activity is measured either in plasma or in whole blood after deproteinization in perchloric acid, but the values obtained with both methods are usually identical because concentration and isotopic equilibration across the red blood cell membrane of mammals seems to be

achieved very rapidly. Unlike most mammalian erythrocytes, fish red blood cells retain thei: nuclei and mitochondria and are capable of aerobic metabolism (Pesquero *et al.*, 1994) with exogenous glucose being the primary source of fuel (Sephton and Driedzic, 1994). However, rainbow trout erythrocytes exhibit low rates of glucose uptake from plasma (Bolis *et* ι 1971; Tse and Young, 1990). Hence, during a continuous infusion of labeled glucose, it is not clear whether glucose specific activity is identical in plasma and inside the red blood cell. Therefore, my goal was to test the two hypotheses listed below:

1. The continuous infusion method yields accurate estimates of *in vivo* glucose turnover rate in rainbow trout, and

2. Glucose flux can be estimated by measuring specific activity in either plasma or whole blood.

MATERIALS AND METHODS

Animals

Rainbow trout of both sexes (469 to 1060 g) were acquired from Linwood Acres Trout Farm (Campbellcroft, Ontario) and held in 550-liter flow-through tanks at 15°C. They were kept in dechloraminated, well oxygenated Ottawa tap water under 12 h light: 12 h dark photoperiod. The animals were acclimated to these conditions for at least 1 month before running experiments and they were fed Purina trout chow 3 times a week until satiation.

Surgical procedure

A double cannulation of the dorsal aorta was done as described in chapter 2. Once this procedure was complete, a ventral incision was used to expose the abdominal cavity. This incision also created a small opening in the pericardial membrane in order to monitor heart rate throughout the experiment. The hepatic portal vein, hepatic vein and coeliac artery were ligatured, and the liver was removed and weighed. Liver weight was later subtracted from body mass for turnover rate calculations.

Experimental protocol

a) <u>Artificial glucose production (AGP):</u>

The infusate was prepared daily by drying an aliquot of 6-^{3}H-glucose (New England Nuclear 1.6 Tbq/mmol or Amersham 1.11 Tbq/mmol) under N_2 and redissolving it in Cortland saline containing 240 mM unlabelled glucose. Exact infusion rate was

determined for each experiment by counting an aliquot of the infusate. The concentration of unlabelled glucose added to the saline at an infusion rate of 1ml·h^{-1} was equivalent to a pump glucose entry rate of 4 µmol min^{-1}. Because the same absolute infusion rate was used for all animals, mass specific infusion rates varied from 4.0 to 6.8 µmol kg^{-1} min^{-1} and averaged 5.1 ± 0.3 µmol kg^{-1} min^{-1}.

With the animal lying in a surgical rack, infusion was started with a calibrated syringe pump at 1 ml·h^{-1} (Chapter 2) (Harvard Apparatus, South Natick, MA). Prior to the start of the infusion, a priming dose equivalent to 120 minutes of infusion was injected. Throughout the infusion, fish were maintained under the same level of anesthesia by continuously irrigating the gills with chilled 15°C water (Gilson water bath) containing buffered MS-222 at the concentrations previously mentioned (Chapter 2). Infusion rates of the radioisotope ranged between 33,841 and 96,928 DPM kg^{-1} min^{-1}. Blood samples (600 µl each) were drawn after 30, 40, 50 and 60 min of infusion.

b) No artificial glucose production (NAGP):

In another group, infusate preparation was done without adding the 240 mM of unlabeled glucose to the solution. Therefore, a continuous infusion of 6-^{3}H-glucose was done following the hepatectomies but without artificially replacing the glucose production with a pump.

Sample analysis

In AGP, each blood sample was immediately separated into two fractions: 300 µl were centrifuged, and 300µl were diluted in 500µl perchloric acid (8%) and centrifuged. After separation, determination of glucose concentration was performed spectrophotometrically (Milton Roy, Spectronic 1001 plus) in plasma and perchloric acid extract at 340 nm via standard enzymatic methods (Bergmeyer, 1985). Plasma glucose activity was measured by drying 30 µl of plasma in an oven at 50 to 60 °C to remove ^3H_2O. Perchloric acid extracts were dried as described in Chapter 2. Once desiccated, plasma and perchloric acid residues were resuspended in 1 ml of water before adding 10 ml ACS II scintillation fluid (Amersham, Oakville Ontario). Counting was performed on a Tri-Carb 2500 scintillation counter (Packard, Canada) with external quench correction. Glucose turnover rate (R_t) was calculated with the steady-state equation of Steele (1959) using either plasma (AGPpl) or whole blood (AGPwb) glucose concentration and activity. In NAGP, determination of glucose concentration and activity was only done on plasma.

Statistics

Differences in glucose concentration, specific activity and turnover rate between the AGPpl, AGPwb and NAGP treatments and between sampling times were assessed with a two-way ANOVA with replication. Differences between the glucose turnover rates of the AGPpl or AGPwb treatments, and the artificial pump glucose entry rate of unlabelled glucose at a given sampling time were assessed by using a t-test. All values are presented as means ± SEM.

RESULTS

Priming dose and time allowed prior to sampling were sufficient to attain concentration and isotopic steady-state (Figure 3.1, $P>0.10$). Results for AGP group are presented in Table 3.1. Heart rate averaged 60 ± 1.3 bpm and did not vary during the experiment (one-way ANOVA; $P=0.31$).

In AGP, AGPpl and AGPwb glucose concentration were significantly different (Figure 3.1a)($P<0.05$). Glucose specific activity and turnover rate estimated for AGPpl and AGPwb were not different (Figure 3.1bc)($P>0.05$). Furthermore, AGPpl and AGPwb glucose turnover rates were not different from the actual unlabelled glucose pump production rate (Figure 3.1 c)($P>0.05$). In Table 3.1, mass specific pump production rates (R inf) were not correlated with the percent error made with glucose turnover rates measured with plasma ($r^2=0.01$; $P=0.95$) or whole blood ($r^2=0.13$; $P=0.50$).

In NAGP, glucose concentration, specific activity and glucose turnover rate averaged 2.7 ± 0.2 mM, 120,900 ± 40,884 DPM μmol^{-1} and 1.1 ± 0.1 $\mu mol\ kg^{-1}\ min^{-1}$, respectively (Figure 3.1). These results were different from glucose concentration, specific activity and turnover rate measured in AGPpl and AGPwb ($P<0.05$).

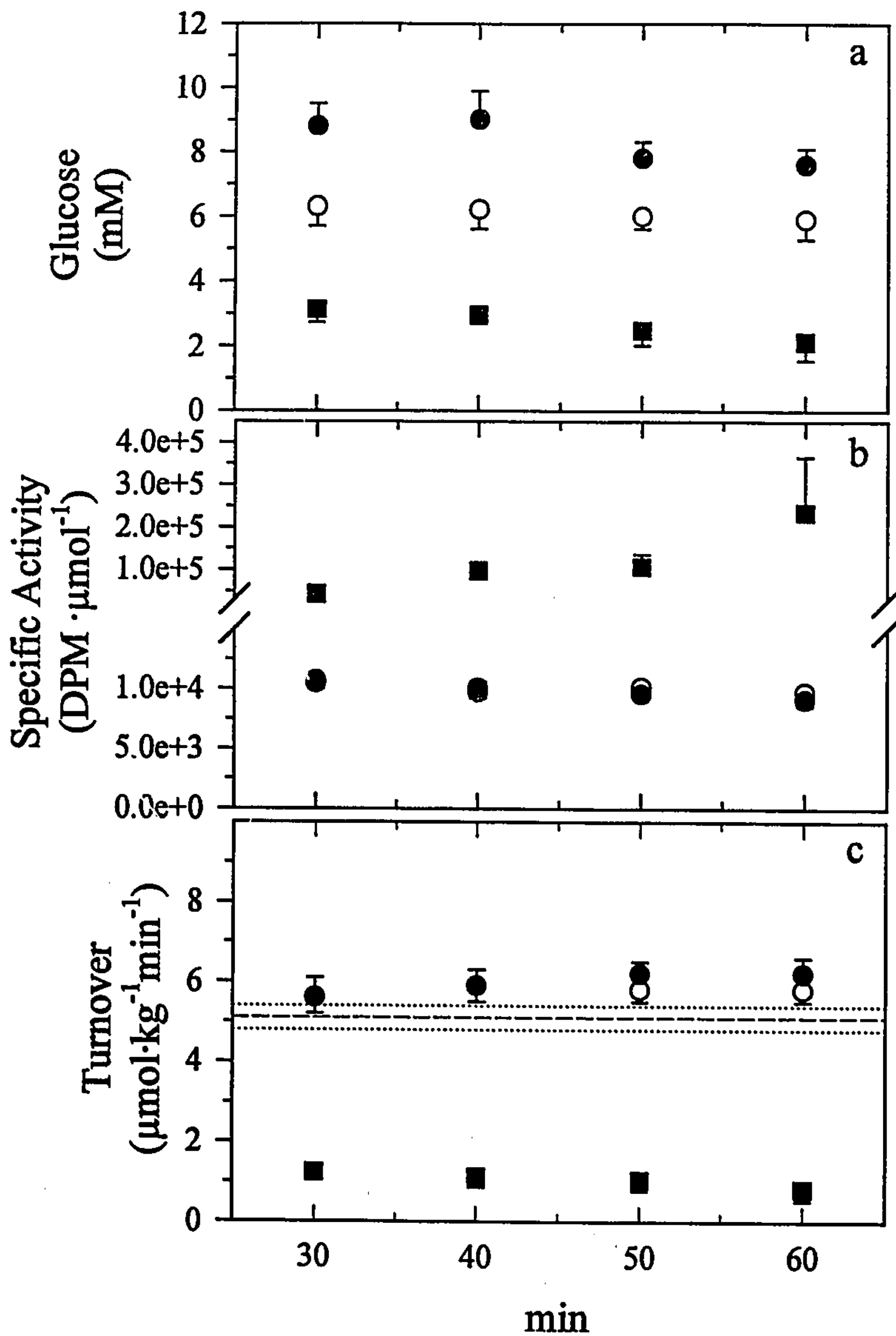
Table 3.1. Plasma ($[Glu]_{plasma}$) and whole blood ($[Glu]_{wb}$)glucose concentrations, specific activity for plasma ($S.A._{plasma}$) and whole blood ($S.A._{wb}$) and turnover rate measured with plasma ($R_{t\ plasma}$)and total blood ($R_{t\ wb}$) in anesthetized, hepatectomized, rainbow trout (15°C) during continuous infusions of 6-^{3}H-Glucose. Infusion rate of unlabelled glucose (R_{inf}), body mass (M_b) and percentage of error* made on the measurement of R_{inf} for plasma (% error plasma) and total blood (% error wb) are also indicated. Within each experiment mean values ± SEM (n=4 blood samples at 30, 40, 50 and 60 min of infusion) are indicated. * % error = $[((R_{t\ plasma}$ or $R_{t\ wb}) - R_{inf}) / R_{inf}] \times 100$

#	M_b (g)	$[Glu]_{plasma}$ (mM)	$[Glu]_{wb}$ (mM)	$S.A._{plasma}$ (DPM·µmol^{-1})	$S.A._{wb}$ (DPM·µmol^{-1})	R_{inf} (µmol·kg^{-1}·min^{-1})	$R_{t\ plasma}$ (µmol·kg^{-1}·min^{-1})
1	854	6.6 ± 0.2	4.2 ± 0.1	7,742 ± 325	9,457 ± 149	4.7	6.8 ± 0.2
2	777	8.3 ± 2.7	6.2 ± 1.5	11,074 ± 432	10,781 ± 174	5.1	5.5 ± 0.2
3	723	7.9 ± 0.2	6.6 ± 0.1	9,401 ± 341	9,994 ± 473	5.5	7.0 ± 0.2
4	592	8.6 ± 0.4	6.4 ± 0.2	10,916 ± 327	10,906 ± 87	6.8	7.3 ± 0.2
5	854	8.0 ± 0.2	7.1 ± 0.4	9,336 ± 205	9,473 ± 454	4.7	5.9 ± 0.1
6	863	9.4 ± 0.5	6.9 ± 0.3	10,908 ± 168	11,465 ± 377	4.6	5.0 ± 0.1
7	1012	8.5 ± 0.3	5.7 ± 0.3	8,938 ± 288	9,039 ± 326	4.0	5.2 ± 0.2
Average	811	8.2	6.2	9,759	10,159	5.1	6.1
SEM	50	0.3	0.4	474	341	0.3	0.4

Table 3.1 (Continued)

$R_{t\,wb}$ ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)	% error plasma	% error wb
5.6 ± 0.1	+ 44.7	+ 19.1
5.7 ± 0.1	+ 7.8	+ 11.8
6.6 ± 0.3	+ 27.3	+ 20.0
7.3 ± 0.0	+ 7.4	+ 7.4
5.8 ± 0.1	+ 25.5	+ 23.4
4.8 ± 0.1	+ 8.7	+ 4.3
5.2 ± 0.2	+ 30.0	+ 30.0
5.9	+ 21.6	+ 16.6
0.3	5.4	3.5

Figure 3.1. Plasma (closed symbols) and whole blood (open symbols) concentration (a), specific activity (b), and glucose turnover rate during primed continuous infusion of 6-^{3}H-glucose for AGP group (closed circles: APGpl; open circles: AGPwb)(n=7) and NAGP group (closed squares)(n=5). Values are means ± SEM. Dashed line represents average mass specific pump production rate and dotted lines SEM.



a
b
c
Glucose (mM)
Specific Activity (DPM ·µmol-1)
Turnover (µmol·kg-1min-1)
min
30
40
50
60

DISCUSSION

Measured plasma and whole blood glucose turnover rates were not different from actual mass specific pump glucose entry rate (Table 3.1). Therefore, these results indicate that the primed continuous infusion technique proposed in chapter 2 is accurate in measuring *in vivo* glucose turnover rate in trout. In addition, the turnover rate can be estimated accurately from specific activity measured either in plasma or whole blood. Mass specific pump glucose entry rate was not correlated with the measured percentage of error for glucose turnover rate determined with plasma or whole blood. Thus, the percentage of error was independent from the artificial glucose production rate.

Normal glucose levels and steady-state conditions show that the glucose entry rate of the pump chosen in this study did not cause fish to become hyperglycemic. As indicated by heart rate, the cardiovascular condition of the fish did not seem to deteriorate throughout the experiment.

In the AGP group, there was a significant difference between the glucose concentration of the plasma and the erythrocyte (Figure 3.1), however, AGPpl and AGPwb specific activity and turnover rate were not different. These results suggest that the erythrocytes did not discriminate between labeled and unlabelled glucose molecules. We found similar results in preliminary experiments on intact animals where no difference was found between glucose turnover rate measured with plasma or whole blood (data not shown).

In the NAGP group, other organs in the fish with glucose production capabilities, such as the kidney, were unable to maintain glucose concentration (Figure 3.1). Under these conditions, glucose concentration and turnover rate fell to 2.7 ± 0.2 mM and 1.1 μmol kg^{-1} min^{-1}, respectively. It is unclear whether this glucose turnover rate represents the production of glucose from the kidney *in vivo* for intact animals, or if this organ increases its rate of glucose production in an attempt to maintain plasma glucose concentration in hepatectomized animals.

Thus, I have shown that the continuous infusion technique developed in chapter 2 is accurate in estimating glucose turnover rate using whole blood or plasma. In view of the resting glucose turnover rate measured in chapter 2, one question comes to mind: why do rainbow trout support such a high glucose turnover rate?

As previously mentioned, the main glucose producers in rainbow trout are liver and, to a lesser extent, kidney. However, the glucose sources available to these tissues are very limited due to dietary limitations. Trout diet, like most carnivorous diet, is usually characterized by a high protein and low carbohydrate composition (Cowey *et al.*, 1977). Furthermore, complex carbohydrates are not very efficiently assimilated by many fish (Cowey *et al.*, 1977). Therefore, gluconeogenesis from amino acids, especially alanine, becomes an extremely important pathway to synthesize glucose and replenish glycogen stores (Bever *et al.*, 1981). It is important to note, that gluconeogenic precursors may vary depending on the conditions faced by the animal (i.e., lactate accumulation due to hypoxia or exercise or alanine influx after a meal). At rest, the Cori cycle was found to operate slowly in the American eel (*Anguilla rostrata*) (Cornish and Moon, 1985) and in

tuna (*Katsuwonus pelamis*) (Weber *et al.*, 1986), suggesting that muscle lactate contributes little to glucose production in fish.

Glycogenolysis and gluconeogenesis are the major pathways for glucose production. If this production was based solely on glycogenolysis, a resting 1kg trout with an hepatosomatic index of 1.0, a liver glycogen content of 120μmol glucosyl units·g^{-1} liver (Wright *et al.*, 1989) and a glucose turnover rate of 9.5 μmol kg^{-1} min^{-1}, would exhaust liver stores in 2 hours. The activity of glycogen phosphorylase, in the active form (GPase), dictates the rate of glycogen breakdown (Wright *et al.*, 1989). Wright *et al.* (1989) and Mommsen and Moon (1990) reported rates of glycogenolysis obtained from isolated hepatocytes. It the first study, percentage of the active form of GPase was measured at 23% of total GPase present in the liver, consequently, glucose production from glycogen was calculated as 2.2 μmol kg^{-1} min^{-1} at 16°C (calculated for a 1 kg fish with a 10g liver). Similarly, the second study reports a rate of glycogenolysis equivalent to 2.4 μmol kg^{-1} min^{-1} at 15°C. Both of these rates represent 13% of the glucose turnover rate measured in chapter 2. If this was the case, the difference (87%) might represent gluconeogenesis which was suggested to be predominant in American eel (Cornish and Moon, 1985) and in kelp bass (*Dicentrarchus labrax*) (Bever *et al.*, 1981). However, biochemists, measuring gluconeogenic rates *in vitro*, face many technical problems when isolating and maintaining hepatocytes at precise temperatures, pH, bicarbonate, ionic and osmotic concentrations. In addition, *in vitro* gluconeogenic rates from lactate or alanine alone tend to be underestimates of those measured using multiple precursors (Suarez and Mommsen, 1987).

In mammals, glucose and free fatty acid oxidation accounts for 30% and 40%, respectively, of total oxygen consumption and 30% coming from endogenous sources (Felig and Wahren, 1975). Comparatively, fish are thought to have a very limited capacity to use carbohydrates (Cowey *et al.*, 1977; Machado *et al.*, 1989; van den Thillart, 1986). Van den Thillart (1986) reported that protein and lipid oxidation represent an important fraction of total energy metabolism in a resting fish while the contribution of carbohydrates is negligible. In fact, estimation from respiratory quotient measurements show that protein and lipid oxidation represent 80% and 20% of energy metabolism, respectively, with 95% coming from endogenous sources. Glucose utilization determined at 15°C with 2-deoxyglucose shows that in a 1 kg trout, 2 to 3% of circulating glucose enters glycolysis in muscular tissues such as the heart, white muscle and red muscle (76% of total body mass) (West *et al.*, 1993). Other tissues like the brain, red blood cells and gonads (2 to 16% of total mass depending on Gonado-Somatic Index) in a 1 kg fish require at most 0 to 2% of circulating glucose for energy metabolism (Washburn *et al.*, 1992). Liver, gills and kidney (2 to 4% of total body mass) have a higher gluconeogenic capacity then the previously mentioned tissues, thus, would metabolize little glucose. Therefore, a minimum of 90 to 95% of circulating glucose is left for other purposes.

Mucus is constantly secreted from the gill surface and the skin. Maintaining this production requires the synthesis of large quantities of glycoproteins (mucopolysaccharides bound to proteins) (Bever *et al.*, 1981). High glucose turnover rates may be explained by these requirements for constant mucus production. A study by Bever *et al.* (1981) determined the incorporation of arterially injected U-^{14}C-alanine and

6-^{3}H-glucose into the skin mucus of kelp bass. The ^{3}H/^{14}C ratio was much lower (0.46) for mucus than for plasma glucose (3.39) suggesting a direct incorporation of ^{14}C into mucopolysaccharides via the alanine gluconeogenesis pathway. An important contribution of glucose to mucus production could also explain the large difference found in glucose turnover between the American eel (high mucus producer) (Cornish and Moon, 1985) and the rainbow trout.

In conclusion, the technique proposed here opens the door to the dynamic study of substrate kinetics under a variety of experimental conditions. In addition to glucose, it can be adapted to the investigation of most metabolic substrates including fatty acids, glycerol, amino acids and lactate. In chapter 4, I will be examining the effects of acute hypoxia and cold exposure on glucose and NEFA kinetics in rainbow trout.

CHAPTER 4

EFFECTS OF ACUTE HYPOXIA AND COLD EXPOSURE ON NEFA AND GLUCOSE KINETICS

INTRODUCTION

The purpose of chapter 2 was to develop a new continuous infusion technique that could be used to study substrate kinetics in fish under a variety of experimental conditions. In chapter 3, this technique was proved accurate to quantify *in vivo* glucose turnover rate. In this chapter, I will look at the effects of rapid environmental perturbations on the release and uptake of two major metabolic fuels. More precisely, I will quantify the effects of acute hypoxia and cold exposure on the fluxes of circulatory glucose and nonesterified fatty acids (NEFA) in rainbow trout. The following hypotheses will be tested: 1) because only carbohydrates can be used in the absence of oxygen, trout exposed to hypoxia will increase their glucose flux and decrease their NEFA flux; 2) during cold exposure, both glucose and NEFA fluxes will decrease in proportion to metabolic rate depression. Background information on the known effects of hypoxia and cold exposure is presented below.

HYPOXIA

Mechanisms and survival strategies during hypoxia have been studied in fish for many years. Some fish (i.e. goldfish, crucian carp, lungfish, eel) survive well in oxygen limited conditions using various physiological and biochemical strategies (Hochachka and Somero, 1984). These oxygen conformers depress their metabolism, use efficient fermentation pathways (i.e., succinate and proprionate vs glucose) and employ better ways of coping with acidic end-product accumulation (i.e., ethanol production, fatty acid elongation) allowing them

to survive long periods in hypoxic and even anoxic water (Hochachka and Somero, 1984). However, other teleosts that find themselves in hypoxic waters try to maintain their metabolic rate, instead of conforming to this new environment. Unfortunately, this permits only short-term resistance and survival is generally compromised when oxygen content of the water is critically low. Rainbow trout is a well studied example of a non-conformer and hypoxia has profound effects on its metabolism.

Metabolic adjustments

The strategy of rainbow trout in an oxygen limiting environment is to offer short-term resistance until the conditions improve. Many teleosts can regulate oxygen extraction over a wide range of oxygen levels in the water. Trout is no exception since it is able to modify its circulatory and respiratory systems to increase the efficiency of gas transfer (Randall, 1982; van Raaij, 1994) allowing a brief resistance to hypoxia (van den Thillart, 1982). These changes (i.e. hyperventilation, increase diffusive conductance and Hb-O_2 affinity/capacity of blood) results from neuronal and hormonal changes during severe hypoxia (Fritsche and Nilsson, 1993; Perry and Wood, 1989). Consequently, this improved capacity to extract oxygen from water will maintain, and in some cases even increase, oxygen consumption depending on the severity of hypoxia (Hughes and Saunders, 1970; McKim and Goeden, 1982; Perry and Wood, 1989; Randall, 1982). Nevertheless, at low oxygen availability, aerobic metabolism is not sufficient to maintain ATP production and a shift toward anaerobic production occurs (Boutilier *et al.*, 1988; Holeton and Randall, 1967; van den Thillart, 1982). In trout, at Pwo_2 between 30 and 50 torr, many researchers have reported

increases in glycogenolysis, blood lactate concentration and lactate turnover rate, all indicative of increased anaerobic glycolysis (Boutilier *et al.*, 1988; Dunn and Hochachka, 1986, 1987; Heath and Pritchard, 1965; Holeton and Randall, 1967; van Raaij, 1994). Consequently, during these oxygen limiting conditions, fuel preference switches from a primarily aerobic substrate (NEFAs and protein) to one used in anaerobic pathways (glucose) (Dunn and Hochachka, 1986; Hochachka and Somero, 1984; Johnston, 1975).

Glucose kinetics

The glucose used to fuel glycolysis originates from two potential sources: 1) *in situ* utilization of local tissue glycogen stores, and 2) supply of glucose from the liver through glycogenolysis and gluconeogenesis, via circulation (Dunn and Hochachka, 1986). In the first case, the size of glycogen stores in tissues may correlate well with their capacity to tolerate hypoxia (Daw *et al.*, 1967; Kerem *et al.*, 1973). In rainbow trout, Dunn and Hochachka (1986) observed that hypoxia caused a decrease in glycogen stores in some tissues (i.e., white muscle, heart, brain) while remaining constant in others (i.e., liver, red muscle). Furthermore, white muscle glycolysis was shown to have a very strong influence on whole body metabolism during oxygen limiting conditions. In fact, this tissue represents a large proportion of total body mass, contains the greatest quantity of glycogen, and produces the largest amount of lactate during hypoxia (Dunn and Hochachka, 1986; Heath and Pritchard, 1965). The lactate produced is readily oxidized by highly aerobic tissues (i.e., red muscle) (Bilinski and Jonas, 1972). Interestingly, hepatic glycogen is not disturbed in oxygen limiting situations suggesting that no mobilization may occur (Dunn and Hochachka, 1986). In the only study

where glucose flux was measured before and during hypoxia, Dunn and Hochachka (1987) showed there was no effect. Unfortunately, the technique used by Dunn and Hochachka (1987) to measure glucose turnover rate only allowed steady-state measurements (bolus injection) giving no information of the release and uptake of glucose occurring during and immediately after the graded hypoxia. Glucose turnover rate remained steady after exposing trout at a Pwo_2 of 30 torr for 3 hours (Dunn and Hochachka, 1987). However, glucose concentration data are somewhat contradictory. In some instances, hypoxia had no effect on glycaemia (Dunn and Hochachka, 1987) while in others, hypoxia caused hyperglycemia (Heath and Pritchard, 1965; Plitsetskaya, 1980; van Raaij, 1994). Severe hypoxia generally resulted in the release of catecholamines (for review see Randall and Perry 1992) and cortisol (van Raaij, 1994; White and Fletcher, 1989) which stimulates glycogenolysis and gluconeogenesis in teleost liver (Ince and Thorpe, 1977; van Raaij, 1994; Wright *et al.*, 1989). In fact, Wright *et al.* (1989) reported that *in vivo* catecholamines administration also resulted in hyperglycemia with no effect on hepatic and muscle glycogen levels.

NEFA kinetics

The literature offers little information on lipid dynamics in hypoxic fish (van Raaij, 1994). In these studies, only plasma NEFA levels were monitored in oxygen limiting conditions and results are somewhat conflicting. Plitsetskaya (1980) reviewed the effects of various stresses including hypoxia, on plasma NEFA concentration of fish. Hypoxic trout, tench (*Tinca tinca*) and lamprey (*Lampetra fluviatilis*) were reported to increase NEFA plasma levels, while hypoxic carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) decreased their

NEFA concentration (Plitsetskaya, 1980; White and Fletcher, 1989). In mammals, adrenergic stimulation increases NEFA levels by stimulating lipolysis (Smith, 1983). However, through inhibition of lipolysis or stimulation of NEFA reesterification, Randle *et al.* (1963) proposed that hyperglycemia could inhibit mobilization of NEFA independently of hormonal stimulation. More recently, Carlson *et al.* (1991) reported that hyperglycaemia caused a decrease of NEFA turnover rate during pancreatic-pituitary clamps. In exercising goats, McClelland *et al.* (1995) also suggested that an inhibition of NEFA mobilization was the result of a high plasma glucose concentration. In fish, Van Raaij *et al.* (1994) suggested that the decrease in plasma NEFA concentration found in trout and carp during hypoxia/anoxia, is not caused by elevated glucose concentrations but by an insulin stimulated lipogenesis. Even though plasma NEFA concentration may yield some qualitative information about the fluxes of this fuel in trout, flux and concentration changes do not necessarily occur in parallel (Weber and Zwingelstein, 1995). Therefore, direct measurement of NEFA turnover rate is absolutely necessary to understand the metabolism of this aerobic fuel during hypoxia. Therefore, the purpose of this part of my study is to determine the effects of acute hypoxia on glucose and NEFA kinetics using a continuous infusion of 6-^{3}H-glucose and 1-^{14}C-palmitate. This technique allows nonsteady-state measurements, thereby providing the opportunity to detect a possible imbalance between the rates of appearance and disappearance of these metabolites.

TEMPERATURE

Even though water has a high thermal capacity, fish experience modifications in ambient temperature at various times (e.g., vertical and spawning migration, seasonal and diurnal changes, global warming). When possible, escape will be the first line of defense, allowing the animal to return to a preferred temperature (for review see Fry, 1971; Kelsch and Neill, 1990; Cherry *et al.*, 1975; Bryan *et al.*, 1990). However, fish may become trapped in cold water and aquatic ectotherms have virtually no physiological and biochemical defenses to resist a drop in their body temperature (Clarke, 1993). Various aspect of temperature acclimation have been studied intensively (for review see Hochachka and Somero, 1984; Guderley, 1990; Clark and Levy, 1988; Crawshaw and Hazel, 1984; Hazel, 1984; Hazel and Prosser, 1974). In particular, compensatory mechanisms such as homeoviscous adaptations of membranes, changes in gene expression and mitochondrial density are relatively well understood. However, very little information is available on the effects of acute temperature change on the regulation of circulatory fuels. Temperature has a major impact on reaction rates through diffusion of metabolic substrate and direct thermodynamic effect (Clarke, 1993). Physiological and biochemical problems associated with acute cold exposure in fish include perturbations in membrane permeability and transport (for review see Hazel, 1973; Hendersen, 1987), catalytic rates of enzymes (for review see Hochachka and Somero, 1984; Sidell and Hazel, 1984; 1987; Wojcieszyz, 1981) and diffusive processes of metabolites and respiratory gases within the tissues (Sidell and Hazel, 1987) compromising cell integrity and function (Hendersen, 1987).

The aim of this part of the study will be to investigate the effects of an acute temperature drop on glucose and NEFA metabolism. More precisely, I will look at the consequences of a 10°C lowering in water temperature on the release and uptake of circulating glucose and NEFA.

MATERIALS AND METHODS

Animals

Rainbow trout of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario), and held in 550-liter flow-through tanks. They were kept in dechloraminated, well oxygenated Ottawa tap water under 12h:12h light:dark photoperiod. The animals were acclimated to these conditions for at least 1 month before running the experiments and they were fed Purina trout chow 3 times a week until satiation. During hypoxia experiments, trout (777 to 1050 g) were acclimated at 10°C. During cold exposure experiments, the animals weighed from 558 to 969g and were acclimated at 15°C.

Surgical procedure

In the hypoxia and cold exposure experiments, surgical procedure and cannulation were done as described in Chapter 2.

Choice of radioactive tracer

Glucose turnover rate was estimated using 6-^{3}H-glucose as tracer. Reasons for choosing this radioactive tracer are mentioned in Chapter 2. Fatty acid turnover rate was estimated with 1-^{14}C-palmitate (16:0). Selection of this fatty acid to represent changes in total NEFA flux raises some concern, since the fractional turnover rate of each individual NEFA is not necessarily the same. Ideally, the NEFA used as tracer must not receive

preferential mobilization and oxidation, it must receive a similar treatment as total NEFA (Spitzer, 1975). Recently, a study in humans by Connor *et al.* (1996) indicated that mobilization of fatty acids into plasma is not proportional to their content in adipose tissue, but rather influenced by their molecular structure. Due to their abundance in human plasma, palmitic and oleic acid have traditionally been chosen as tracers (Hagenfeldt, 1975). In dogs (*Canis familiaris*) and goats (*Capra hircus*), by calculating the coefficient of variation of the changes in percent composition of each plasma NEFA, McClelland *et al.* (1995) showed that fluctuations in palmitic and oleic acids concentrations are the most representative of total NEFA concentration changes during exercise. Therefore, these two fatty acids should be the preferred tracers in these animals (McClelland *et al.*, 1995). However, this conclusion may not apply to all animals. Although, interpretation of total NEFA flux from a single fatty acid tracer may not be fully accurate (Weber and Zwingelstein, 1995), it constitutes a good starting point. As in the study of McClelland *et al.* (1995), coefficients of variation will be calculated using the fractional contribution of individual NEFAs to total NEFA concentration during hypoxia and cold exposure.

Preparation of the infusate and continuous isotope infusion

Plasma was collected from a sacrificed animal and used as a source of lipid-binding protein to incorporate 1-^{14}C-palmitate to the infusate. The infusate was prepared daily using 6-^{3}H-glucose (New England Nuclear 1.6 TBq/mmol or Amersham 1.1 TBq/mmol) and 1-^{14}C-palmitate in toluene (Amersham 1.85-2.2 GBq/mmol). An aliquot of 1-^{14}C-

palmitate was dried under N_2 and resuspended by carefully washing the side of the eppendorf tube with 30-50 µl of ethanol. Ethanol and 1-^{14}C-palmitate mixture was then injected in 600 µl of plasma constantly agitated. The pipette tip and the wall of the eppendorf were rinsed repeatedly to recuperate the maximum of activity. This solution was then mixed to a 6-^{3}H-glucose infusate prepared as mentioned in Chapter 2.

After surgery, fish were recovered in opaque Plexiglas boxes irrigated with either dechloraminated, well oxygenated 10°C (hypoxia treatment) or 15°C (cold exposure treatment) tap water. Hematocrit was measured prior to the start of the continuous infusion and only trout with normal values (Hct>20%) were used for the experiments. As the animal was resting in an opaque chamber, a continuous infusion of 6-^{3}H-glucose and 1-^{14}C-palmitate at 1 ml/h was started using a calibrated syringe pump (Harvard Apparatus, South Natick, MA). Prior to the start of the infusion, a priming dose of 6-^{3}H-glucose equivalent to 90 min of infusion was injected. During hypoxia experiments, infusion rates ranged from 156 532 and 368 977 DPM kg^{-1} min^{-1} for glucose and from 25 667 to 300 488 DPM kg^{-1} min^{-1} for palmitate. During cold exposure experiments, infusion rates ranged from 190 670 to 359 588 DPM kg^{-1} min^{-1} for glucose and from 154 561 to 411 090 DPM kg^{-1} min^{-1} for palmitate. In both experiments, steady-state blood samples were taken after 40, 50 and 60 minutes from the beginning of the infusion. A detailed description of the procedure followed during a continuous infusion is given in the previous chapter 2.

Oxygen consumption measurements

During hypoxia and cold exposure experiments, oxygen consumption was determined as described in chapter 2. However, measurement frequency varied with the experiment and exact times are given in the individual sections of the experimental protocol.

Experimental protocol

During hypoxia experiments, following steady-state measurements, oxygen content of the Plexiglas chamber decreased over a period of 20 min using nitrogen bubbling through a column of marbles. Additional blood samples (400 µl each) were collected when water partial oxygen pressure reached 60 torr (38% saturation) and 40 torr (25% saturation) followed by three subsequent samples collected every 30 minutes from the time 40 torr was reached. Oxygen consumption was measured during normoxia and 115 min after the beginning of hypoxia.

During cold exposure experiments, water supply was switched from the 15°C to a 5°C tank following steady-state measurements. The decrease in water temperature was manually controlled to reached 5°C in 20 min. Additional blood samples (400 µl each) were collected when water temperature reached 10°C and 5°C followed by three subsequent samples collected every 30 minutes from the time when 5°C was reached. Furthermore, oxygen consumption was measured at 15, 33, 50, 105, 140 and 173 min.

In both hypoxia and cold exposure experiments, water temperature, oxygen content and percent oxygen saturation were monitored using a temperature and oxygen electrode (Oxyguard, Handy Mk III, Valox ltd). Total sampled blood did not exceed 10% of the animals' blood volume. Blood samples were centrifuged immediately, plasma was separated and placed on ice. Following the experiment all samples were frozen at -20°C until they were analyzed.

Sample analysis

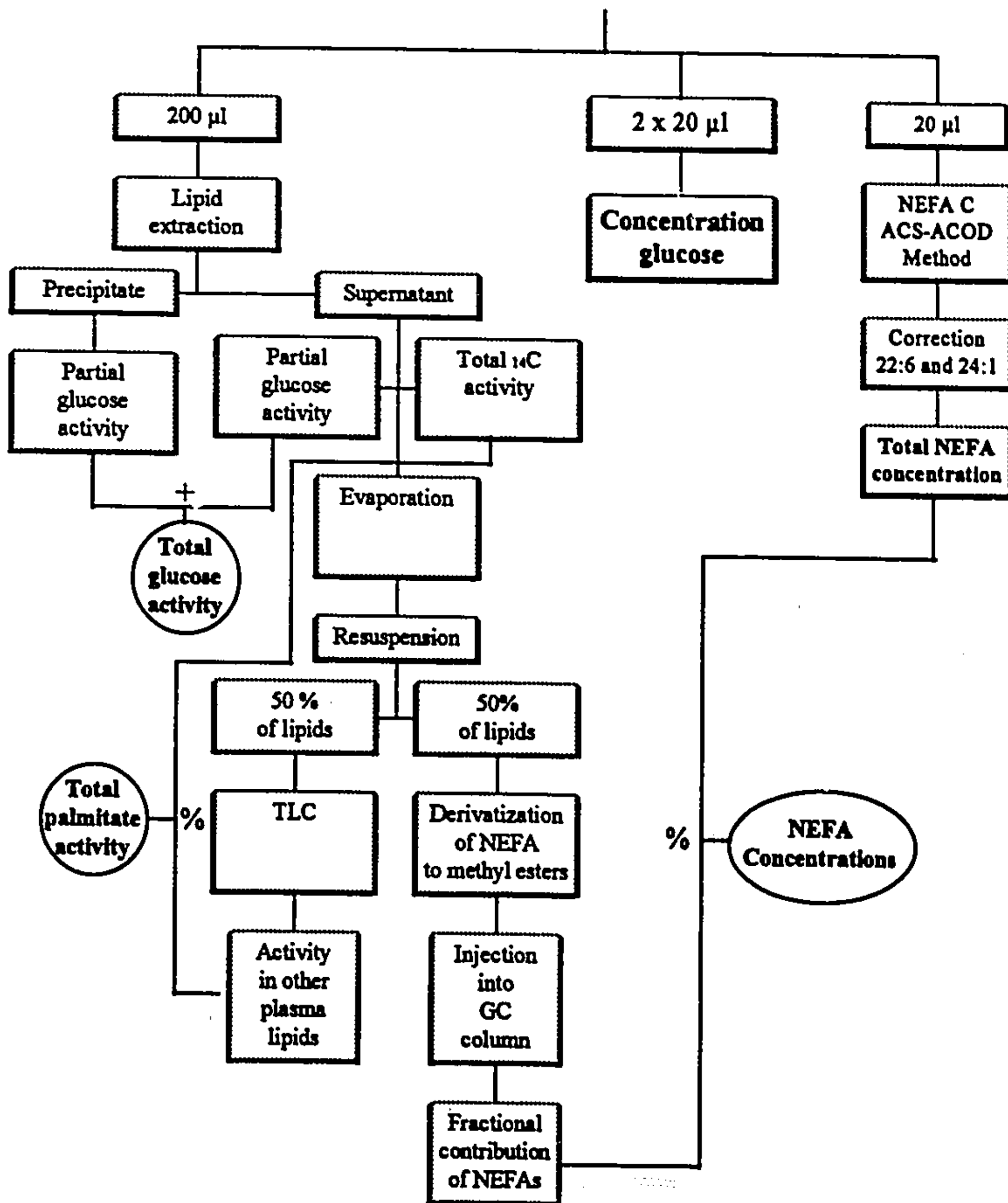
Analytical procedure and sample repartition for hypoxia and cold exposure experiments are described in Figure 4.1. Glucose concentration was measured at 340 nm using a standard enzymatic method (Bergmeyer, 1985) on a Beckman spectrophotometer (DU 640). Total NEFA concentration was measured by an enzymatic colorimetric method with the WAKO test-kit (NEFA C method, Wako Pure Chemical Industries, Osaka, Japan). However, when this test-kit was used to determine the concentration of a 22:6 standard, 22:6 concentration was underestimated by 25%. Since 22:6 represents an important fraction of total NEFA concentration (20-25%), the fraction of 22:6 that was undetected by the kit was added to total NEFA concentration.

The fractional importance of individual NEFAs to total NEFA concentration was determined using a Hewlett Packard (5890 series II) gas chromatographer (GC). Prior to injection into the column, NEFAs were extracted from plasma and methylated using a modification of the method of Tserng *et al.* (1981) described in McClelland *et al.* (1995).

Extraction was done by mixing 200µl of plasma with 80µl 100mM/1N EDTA/HCl, 1:9 v/v and 50µl margaric acid (17:0) (internal standard, 0.6 mg/ml isopropanol). After being vortexed and centrifuged, the mixture was injected into a 50 ml tube containing 28 ml hexane:isopropanol (3:2 v/v) and hand mixed very strongly for 20 sec before being set aside for 10 minutes. The mixture was then centrifuged at 2500 rpm in a Beckman (CS-6) centrifuge for 10 minutes. The supernatant was decanted into a 100 ml pear-shaped flask and an 1 ml aliquot was combined with 10 ml of ACS-II to measure total ^{14}C activity and partial glucose activity (^{3}H). The supernatant left in the 100 ml pear-shaped flask was set aside and used in the methylation of NEFAs described below. The precipitate found at the bottom of the 50 ml tube was dissolved in 1 ml 1N NaOHand 10 ml distilled water. Aliquots of 500 µl were then combined to 10 ml of ACS-II in 20 ml scintillation vials. To avoid excess chemiluminescence, the solution in each scintillation vials was acidified with 10µl of acetic acid glacial (17.5 M). All counting was performed on a Tri-Carb 2500 scintillation counter (Packard, Canada) with external quench correction. Activities in the precipitate and supernatant were added to give total ^{3}H activity (^{3}H-glucose and $^{3}H_2O$). Since $^{3}H_2O$ was found to be less than 3% of total activity even after 3 hours of infusion, samples were not dried prior to counting the activity.

The remaining supernatant was evaporated using a Büchi (RE 121) rotovapor at 50°C and 120 rpm, and the residue was then resuspended in 2 ml of chloroform:methanol (2:1). The resulting lipidic solution was split into two parts: one used in the methylation of NEFAs and the other in the thin layer chromatography (TLC).

Figure 4.1. Plasma repartition and analytical procedure used to measure glucose concentration, glucose activity, NEFA concentration and NEFA activity.



Plasma sample
200 µl
2 x 20 µl
20 µl
Lipid extraction
Concentration glucose
NEFA C ACS-ACOD Method
Precipitate
Supernatant
Partial glucose activity
Partial glucose activity
Total 14C activity
Correction 22:6 and 24:1
Total NEFA concentration
+
Total glucose activity
Evaporation
Resuspension
50 % of lipids
50% of lipids
Total palmitate activity
%
TLC
Derivatization of NEFA to methyl esters
%
NEFA Concentrations
Activity in other plasma lipids
Injection into GC column
Fractional contribution of NEFAs

The first fraction was evaporated under a constant stream N_2 at 50°C (all evaporation procedures were done under these conditions) and resuspended in 100 µl methanol. Dimethoxypropane (1 ml) and 11N HCl (40 µl) were then added mixing well between each step. The mixture was then left at room temperature for 20-30 minutes. Before being evaporated to 100 µl, 20 µl pyridine were added and the solution was vortexed. Isooctane (750 µl) and 500 µl distilled water were then added to the solution, violently shaken, centrifuged and then the upper phase of this two phase solution was removed. This operation was repeated a second time to recuperate the maximum methylated fatty acids before being evaporate totally. The residue was dissolved in 50 µl of isooctane and was transferred to a gas chromatography tube. Samples were injected into the gas chromatography column using a Hewlett Packard 7673 autosampler controller. Injection port temperature was set at 220°C and the detector at 240°C. Column temperature was maintained at 185°C for 34 min before being increased (5°C/min) to 210°C for the remaining 11 minutes. Retention times for individual NEFA were identified using reference methyl-NEFA (Nu-Chek-Prep, Inc., Elysian, Minnesota) and PUFA I standards.

Finally, the second lipidic fraction was used to determine distribution of ^{14}C in the different plasma lipids with TLC. The sample was evaporated and the residue was dissolved in 100 µl benzene:methanol (2:1). Silica gel plates 60 F_{254} (Merck) were cut (10 cm x 10 cm) and three spots of 15 µl (5 µl at a time and blow dried with a hair drier) were placed on the TLC plate. The plate was then placed in solvent (heptane:isopropyl ether:acetic acid; 60:40:4) which was left to migrate until it reached the top. It was then

dried and fractions were revealed under iodine and circled with a pencil (Figure 4.2). The iodine was removed using the hair dryer and fractions were individually scraped into 20 ml scintillation vials containing 4 ml ethanol:water (1:1) and 10 ml ACS-II added. After vortexing the solution well, the activity of the different plasma lipids was measured with a β-counter. Total palmitate activity was calculated by multiplying total ^{14}C activity found in the supernatant after the lipid extraction by the percentage of activity found in the NEFA fraction after TLC.

Calculations and statistics

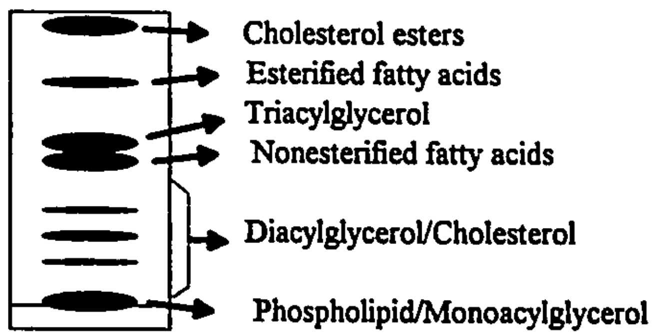
At steady-state, glucose and palmitate turnover rates were calculated using steady-state equation of Steele (1959) (Chapter 2). Total NEFA turnover rate was quantified by dividing palmitate turnover rate by the fractional contribution of palmitate to total NEFA concentration. Glucose and NEFA appearance (Ra) and disappearance (Rd) rates were calculated separately using the nonsteady-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 sampling time at times *1* and *2*. Rates of appearance and disappearance were only plotted if they were significantly different from each other. To assess differences between glucose and NEFA appearance and disappearance rates at a given sampling time, a Bonferonni t-test was used. In tables and

Figure 4.2. Schematic diagram of the migration of different plasma lipids using thin layer chromatography.


Cholesterol esters
Esterified fatty acids
Triacylglycerol
Nonesterified fatty acids
Diacylglycerol/Cholesterol
Phospholipid/Monoacylglycerol

figures, differences in metabolite concentrations, activities and fluxes during hypoxia and cold exposures were assessed using a two-way ANOVA (fish and time). All values are shown as means ± SEM. During cold exposure experiments, Q_{10} values were calculated for oxygen consumption as well as glucose and NEFA turnover rates using the van't Hoff equation:

$$Q_{10} = \frac{Rate^{6^\circ C}}{Rate^{15^\circ C}}^{10/(6^\circ C - 15^\circ C)}$$

The Q_{10} for oxygen consumption was calculated using the steady-state average and the value measured at 173 minutes after the beginning of the infusion. In the case of glucose and NEFA turnover rate, steady-state fluxes and average flux values at 144 and 180 minutes ($P>0.05$) were used for the calculation.

RESULTS

Plasma NEFA concentrations and % composition

As shown in Table 4.1 and Table 4.2, individual NEFA concentrations and fractional contribution to total NEFA concentration of twelve and eleven different NEFAs were measured during hypoxia and cold exposure experiments, respectively. Only NEFAs representing more than 1% of total NEFAs were tabulated. In response to hypoxia and cold exposure, the fractional contribution of individual NEFAs remained unchanged ($P>0.05$). While individual NEFA concentrations did not change ($P>0.05$) during hypoxia experiments, all NEFA concentrations were significantly increased by the end of the cold exposure experiments ($P<0.002$). Mean coefficients of variation (CV) were calculated from the hypoxia and cold exposure experiments using the fractional composition of individual NEFAs to total NEFA concentration (Table 4.3). In comparison to all other measured NEFAs, 16:0 (palmitate) and 22:6 (eicosahexaenoate) had the lowest CVs with respective values of 11.90 and 10.10 during hypoxia experiment and, 8.70 and 11.6 during cold exposure experiment.

Distribution of ^{14}C activity in plasma lipids

During hypoxia and cold exposure experiments, the distribution of ^{14}C activity in different plasma lipids was quantified using thin layer chromatography. The NEFA fraction contained 64.6 ± 0.0 % of total ^{14}C activity during hypoxia and 79.4 ± 0.7 % of total ^{14}C activity during cold exposure. In addition, the ^{14}C activity found in the NEFA fraction was unaffected over time ($P>0.05$).

Table 4.1. Plasma NEFA concentration ([ ]; mM) and fractional contribution of individual NEFA to total NEFA concentration (%) in rainbow trout during normoxia and hypoxia. Mean values (Calculated time and fish) are presented because concentration did not change significantly over time (n=8).

	Normoxia		Hypoxia	
	[ ]	%	[ ]	%
16:0	0.21 ± 0.02	21.2 ± 0.2	0.24 ± 0.00	21.0 ± 0.0
16:1	0.02 ± 0.00	2.4 ± 0.1	0.03 ± 0.00	2.4 ± 0.0
18:0	0.12 ± 0.01	12.4 ± 0.3	0.14 ± 0.00	12.3 ± 0.1
18:1	0.15 ± 0.02	15.3 ± 0.2	0.17 ± 0.00	15.7 ± 0.1
18:2	0.02 ± 0.00	2.5 ± 0.0	0.03 ± 0.00	2.5 ± 0.0
18:3 - 20:1	0.06 ± 0.01	6.2 ± 0.1	0.07 ± 0.00	6.2 ± 0.1
20:2	0.02 ± 0.00	1.6 ± 0.0	0.02 ± 0.00	1.6 ± 0.1
20:3	0.03 ± 0.00	2.7 ± 0.1	0.03 ± 0.00	2.6 ± 0.1
20:4	0.06 ± 0.01	5.8 ± 0.0	0.07 ± 0.00	5.8 ± 0.1
20:5	0.02 ± 0.00	2.6 ± 0.1	0.03 ± 0.00	2.6 ± 0.2
22:5	0.03 ± 0.00	2.7 ± 0.0	0.03 ± 0.00	2.7 ± 0.1
22:6	0.25 ± 0.02	24.9 ± 0.0	0.28 ± 0.01	24.8 ± 0.3

Table 4.2. Effect of cold exposure on plasma NEFA concentration ([ ]; mM) and fractional contribution of individual NEFA to total NEFA concentration (%) in 15°C acclimated rainbow trout (n=8). * NEFA concentration increased significantly after 90 min of cold exposure ($p<0.05$).

	15°C		6°C	
	[ ]	%	[ ]	%
16:0	0.18 ± 0.00	23.6 ± 0.1	0.25* ± 0.02	23.8 ± 0.1
16:1	0.01 ± 0.00	1.7 ± 0.0	0.02* ± 0.00	1.6 ± 0.0
18:0	0.11 ± 0.00	15.4 ± 0.2	0.15* ± 0.01	15.2 ± 0.3
18:1	0.09 ± 0.00	11.3 ± 0.1	0.13* ± 0.01	11.5 ± 0.1
18:2	0.02 ± 0.00	2.5 ± 0.0	0.03* ± 0.00	2.6 ± 0.0
18:3 - 20:1	0.04 ± 0.00	4.6 ± 0.1	0.06* ± 0.01	4.8 ± 0.1
20:3	0.02 ± 0.00	3.1 ± 0.1	0.04* ± 0.01	3.3 ± 0.1
20:4	0.04 ± 0.00	5.6 ± 0.1	0.06* ± 0.01	5.7 ± 0.1
20:5	0.05 ± 0.00	6.2 ± 0.2	0.06* ± 0.01	6.1 ± 0.1
22:5	0.02 ± 0.00	2.2 ± 0.2	0.02* ± 0.00	2.1 ± 0.0
22:6	0.17 ± 0.00	21.9 ± 0.4	0.23* ± 0.02	21.6 ± 0.1

Table 4.3. Mean coefficient of variation for individual NEFA composition (% of total NEFA concentration) throughout hypoxia and cold exposure experiments.

	Hypoxia	Cold exposure
16:0	11.90	8.70
16:1	43.90	28.30
18:0	23.20	20.90
18:1	17.10	13.20
18:2	12.10	27.60
18:3-20:1	13.90	27.50
20:2	41.90	——
20:3	39.80	33.60
20:4	26.85	16.40
20:5	46.40	15.80
22:5	48.90	23.60
22:6	10.10	11.60

HYPOXIA EXPERIMENTS

Oxygen consumption

Oxygen partial pressures in water measured throughout the experiment and rates of oxygen consumption determined at the beginning (normoxia) and end (hypoxia) of the experiment are presented in Figure 4.3. Partial pressure of oxygen in the water decreased from 153 ± 0.9 torr (96% of saturation) to 39 ± 0.2 torr (25% of saturation) in 20 minutes (equivalent to a rate of 6 torr/min) and was maintained at the lower level until the end of the experiment. Oxygen consumption at the end of the experiment was not different than its normoxic value of 41.8 ± 6.1 μmol O_2 kg^{-1} min^{-1} ($P=0.29$).

Glucose concentration and turnover rate

Changes in glucose concentration, specific activity, flux and lactate concentration as fish went from normoxia to hypoxia are plotted in Figure 4.4. Normoxia glucose concentration, turnover rate and lactate concentration were measured at 5.5 ± 0.03 mM, 5.4 ± 0.18 μmol kg^{-1} min^{-1} and 1.1 ± 0.01 mM, respectively. As water was rendered hypoxic, glucose concentration increased by 1.3 fold 30 min after reaching 25% of oxygen saturation ($P<0.001$) and decreased at the end of the experiment. Lactate concentration increased by 6 fold during the experiment, reaching a value of 5.8 ± 0.53 mM ($P<0.001$). Glucose appearance rate was significantly higher than disappearance rate 15 min after reaching 25% of oxygen saturation ($P=0.03$) but came back to normoxic values 140 min from the beginning of the infusion.

Figure 4.3. Mean oxygen consumption in rainbow trout during normoxia and hypoxia (histogramme) (n=8). Modification in PwO_2 indicated as mean (thick line) ± SEM (thin line).

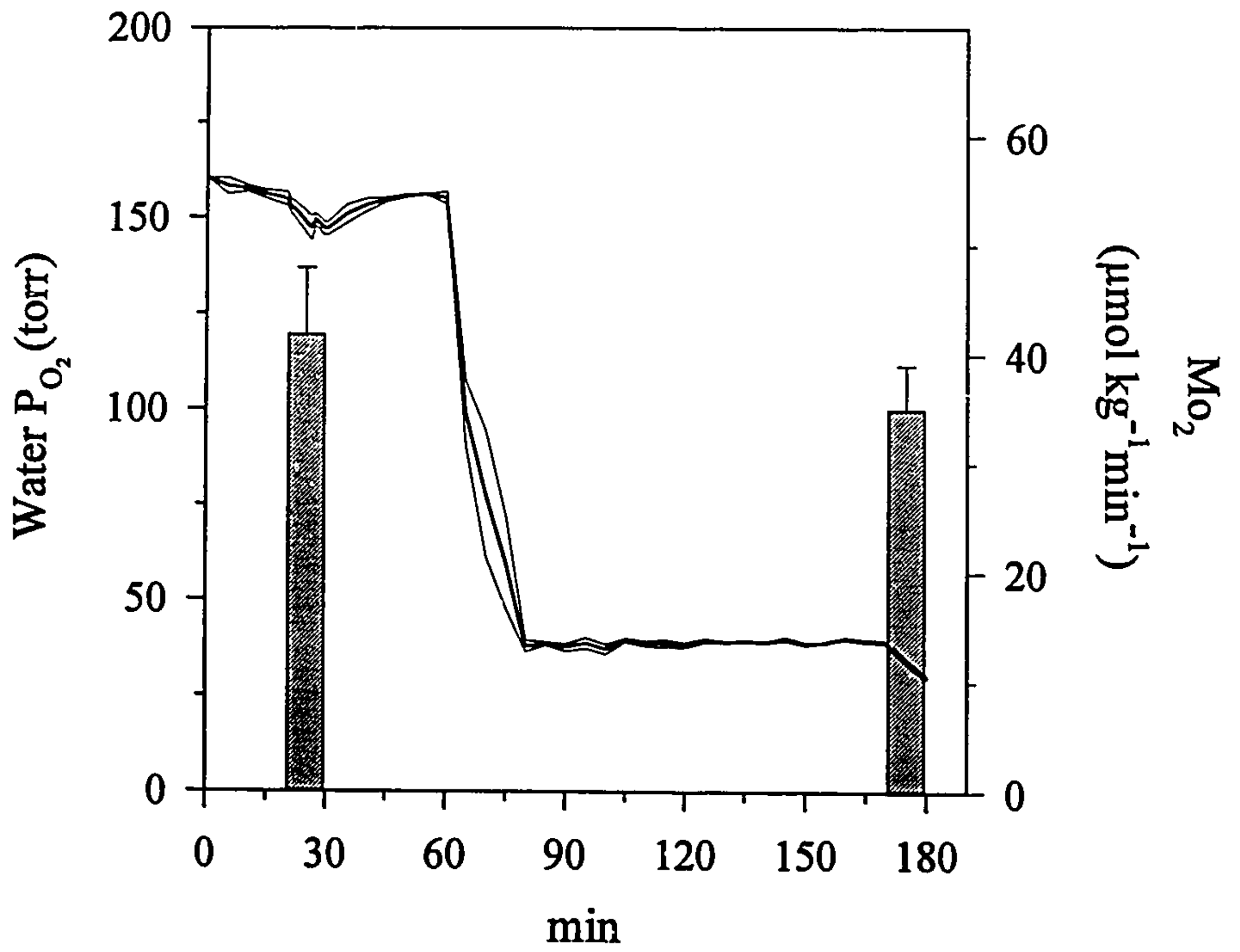
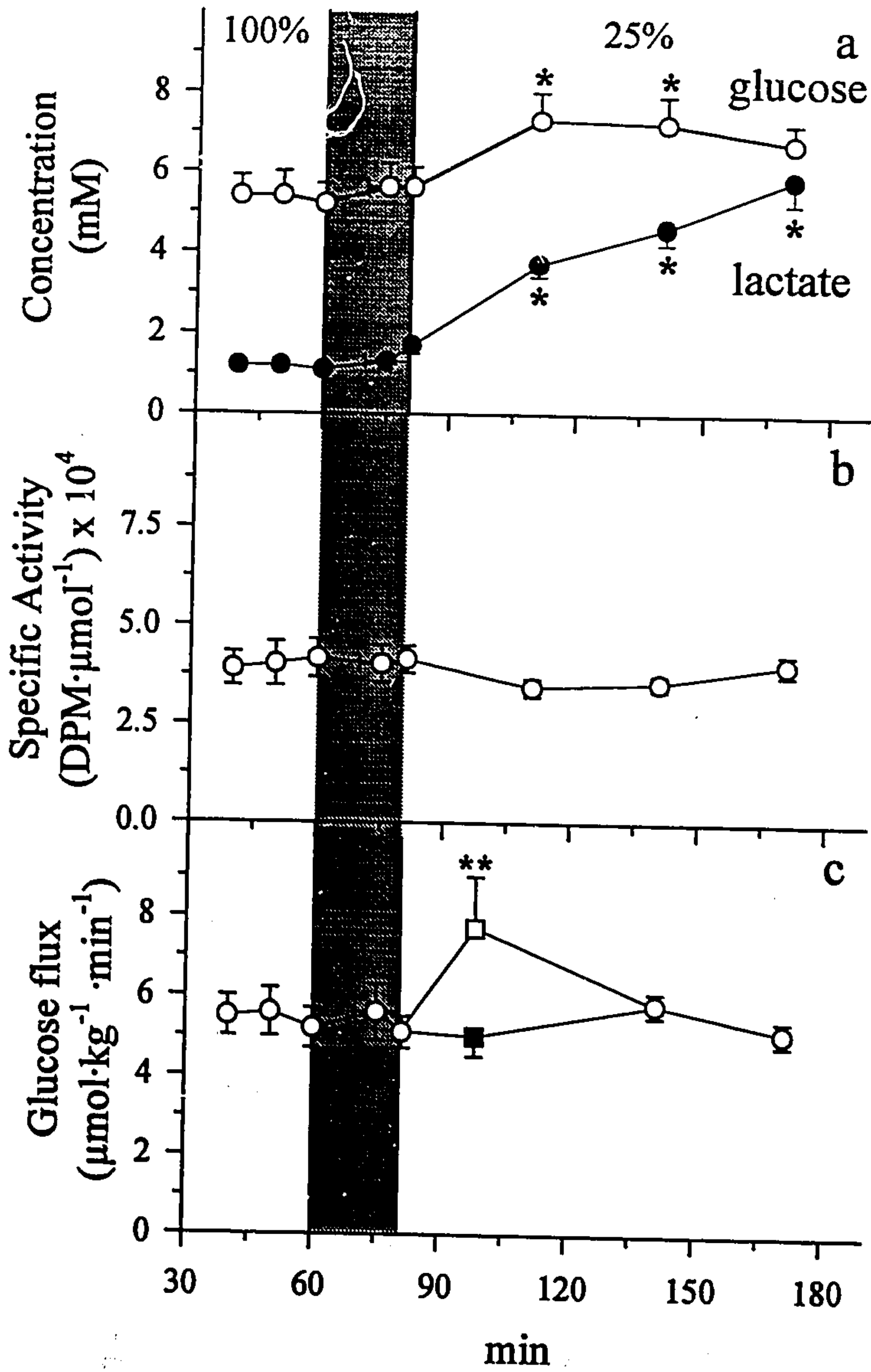

200
150
100
50
0
Water P_{O_2} (torr)
60
40
20
0
$\dot{M}o_2$
(μmol kg^{-1} min^{-1})
0
30
60
90
120
150
180
min

Figure 4.4. Mean plasma glucose concentration (○) (a), lactate concentration (●) (a), glucose specific activity (b) and glucose flux (c) in rainbow trout during normoxia (100%), graded hypoxia (gray zone) and hypoxia (25%)(n=8). * indicates a significant difference from steady-state values ($p<0.05$). ** indicates a significant difference between glucose rate of appearance (□) and disappearance (■)($p<0.05$).


100%
25%
a
glucose
lactate
Concentration (mM)
8
6
4
2
0
b
Specific Activity (DPM·µmol-1) x 10^4
7.5
5.0
2.5
0.0
c
Glucose flux (µmol·kg-1 ·min-1)
30
60
90
120
150
180
min

NEFA concentration and turnover rate

Figure 4.5 shows the effects of hypoxia on palmitate concentration, specific activity and fractional contribution to total NEFA concentration. Palmitate concentration remained at normoxic levels (0.21 ± 0.02 mM) throughout hypoxia ($P>0.05$). Palmitate specific activity changed significantly over time ($P=0.04$), but pairwise comparisons of means could not localize this difference. Fractional contribution of palmitate to total NEFA concentration was not affected by the change in oxygen availability ($P>0.05$). Normoxia total NEFA concentration and turnover rate averaged 0.98 ± 0.10 mM and 5.8 ± 0.4 µmol kg^{-1} min^{-1}, respectively (Figure 4.6). Total NEFA concentration did not vary over time during hypoxia ($P>0.05$). However, NEFA turnover rate decreased significantly after 60 min at 25% oxygen saturation, reaching 53% (3.3 µmol kg^{-1} min^{-1}) of normoxia turnover rate by the end of the experiment ($P=0.007$).

COLD EXPOSURE EXPERIMENTS

Oxygen consumption

Water temperature and oxygen consumption during the cold exposure experiments are presented in Figure 4.7. When water flow was switched from the 15°C to the 5°C tank, water temperature in the Plexiglas box decreased at an approximate rate of 0.5 °C/min reaching a final value of 6°C (5.8 ± 0.04°C) after 20 minutes. Percent oxygen saturation was monitored constantly during the experiment and it remained stable at values nearing full saturation. As a result of the 9°C decrease in temperature, oxygen consumption dropped progressively from 41.2 ± 2.3 µmol kg^{-1} min^{-1} to 21.7 ± 1.4 µmol kg^{-1} min^{-1} by the end of the experiment ($P<0.001$).

Figure 4.5. Mean plasma palmitate concentration (a), specific activity (b) and fractional contribution to total NEFA concentration (c) in rainbow trout during normoxia (100%), graded hypoxia (gray zone) and hypoxia (25%) (n=7).

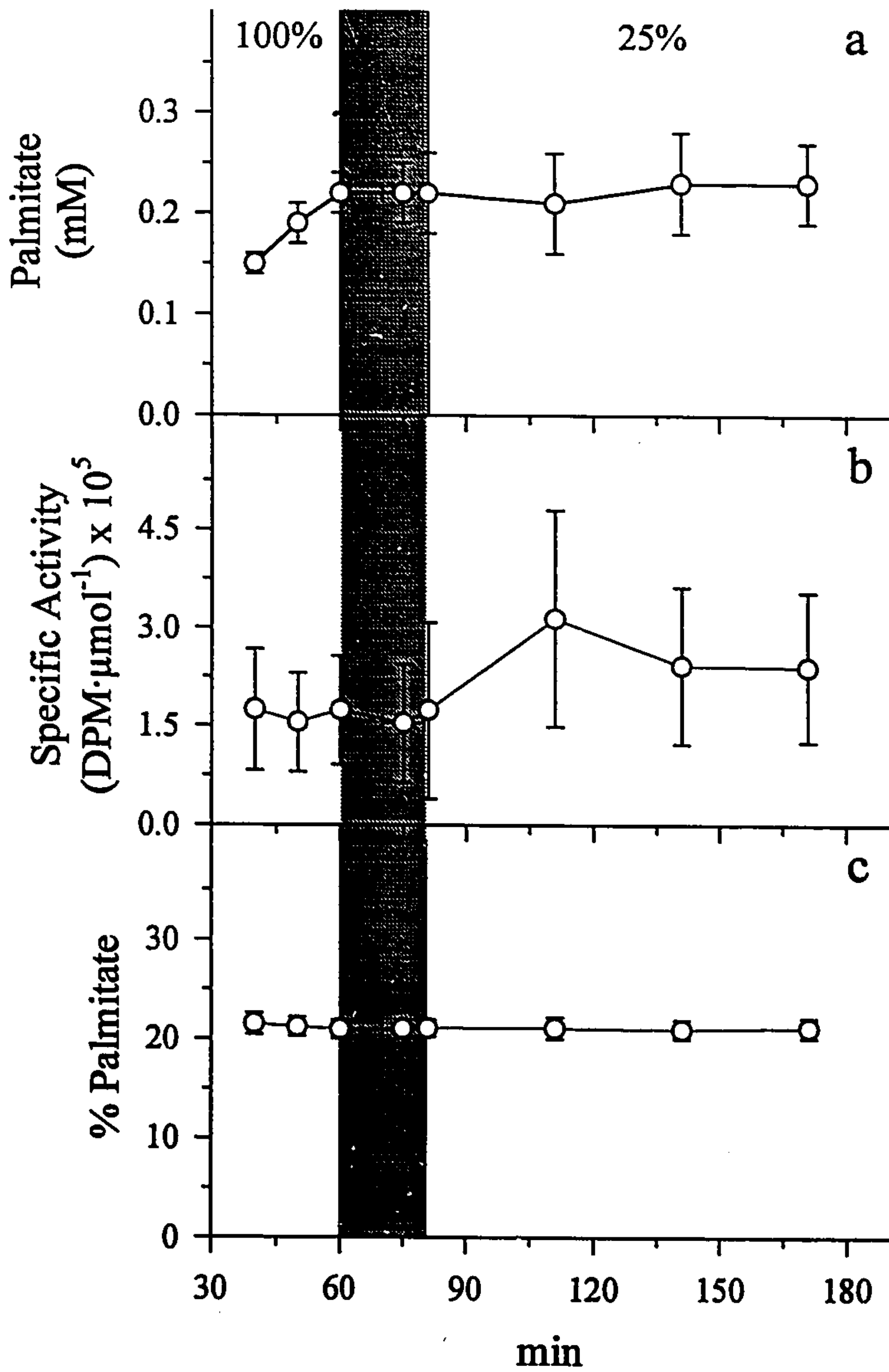
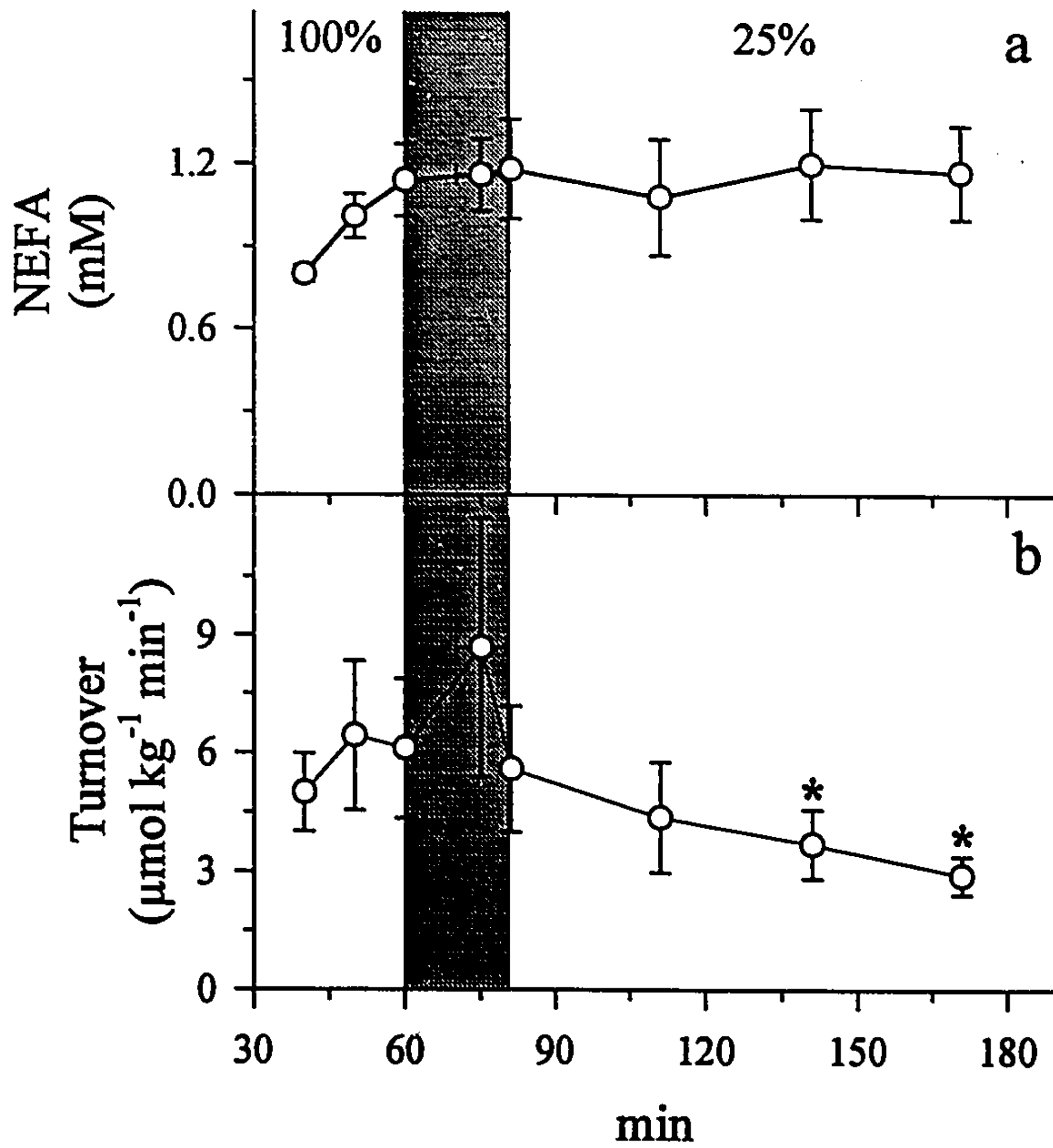
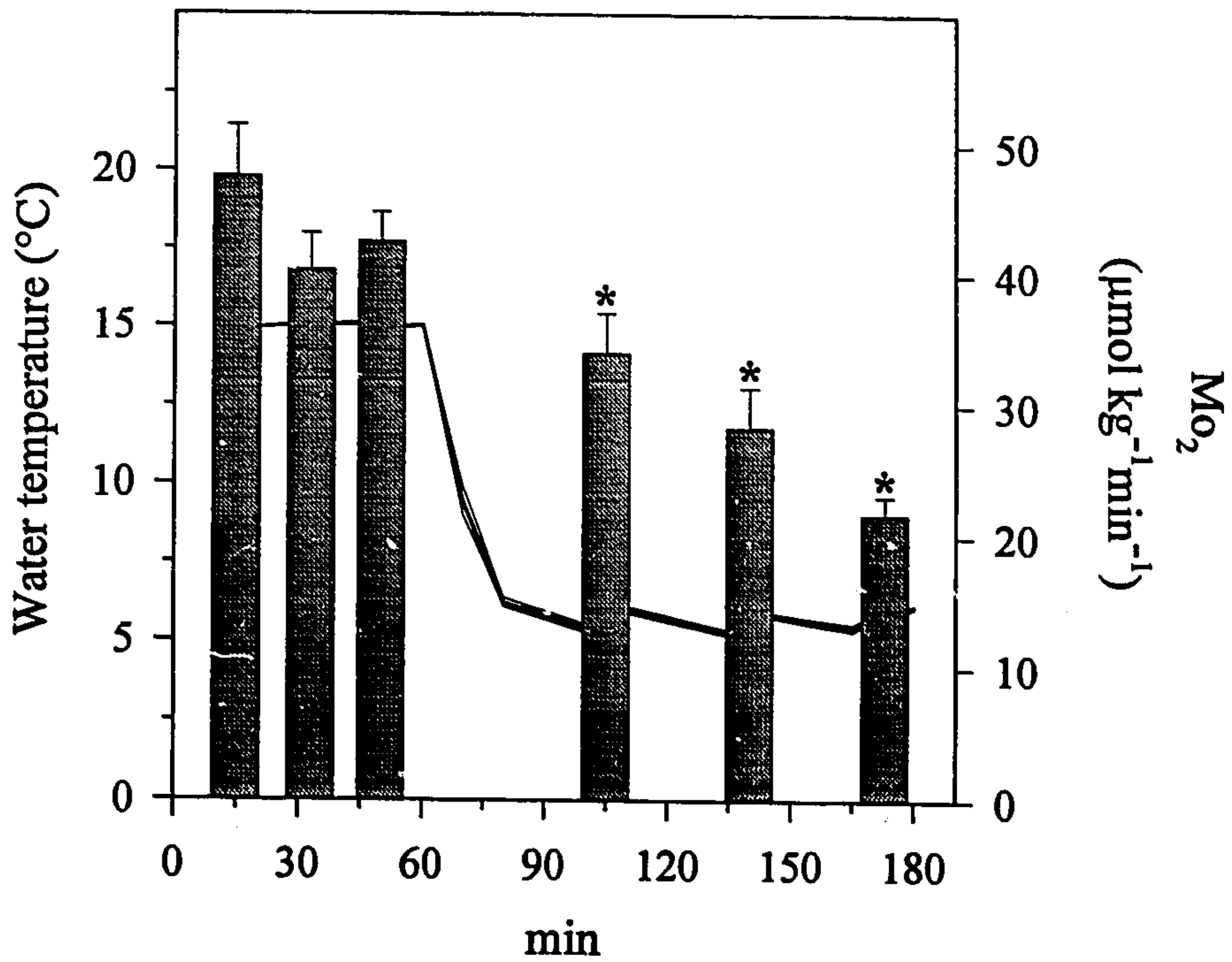

100%
25%
a
b
c
Palmitate (mM)
0.3
0.2
0.1
0.0
Specific Activity (DPM·µmol⁻¹) x 10⁵
4.5
3.0
1.5
0.0
% Palmitate
30
20
10
0
30
60
90
120
150
180
min

Figure 4.6. Mean plasma NEFA concentration (a) and turnover rate (b) in rainbow trout during normoxia (100%), graded hypoxia (gray zone) and hypoxia (25%) (n=7). * indicates a significant difference from steady-state values ($P<0.05$).



100%
25%
a
b
NEFA
(mM)
1.2
0.6
0.0
Turnover
(μmol kg-1 min-1)
9
6
3
0
*
*
30
60
90
120
150
180
min

Figure 4.7. Effect of cold exposure (6°C) on mean oxygen consumption in rainbow trout (15°C acclimation temperature) (histogramme) (n=8). Modification in water temperature indicated as mean (thick line) ± SEM (thin line). * indicates a significant difference from steady-state values ($p<0.05$).


Water temperature (°C)
$\dot{M}o_2$
(μmol kg^{-1} min^{-1})
min
0
5
10
15
20
0
10
20
30
40
50
0
30
60
90
120
150
180
*
*
*

Glucose concentration and turnover rate

Effect of cold exposure on glucose concentration, specific activity and turnover rate is presented in Figure 4.8. Concentration and isotopic steady-state conditions were attained after 40 min of infusion at a glucose concentration of 7.5 ± 0.05 mM and a turnover rate of 8.6 ± 0.12 μmol kg^{-1} min^{-1} (P>0.05). As the temperature was lowered, glucose concentration decreased overall (P=0.01), but pairwise comparisons of means could not localize this difference precisely. Glucose specific activity and turnover rate were significantly altered (P<0.001) 30 minutes after the temperature reached 6°C. Glucose turnover rate decreased from 8.8 ± 1.4 to 4.6 ± 0.7 μmol kg^{-1} min^{-1} by the end of the experiment. Rates of glucose appearance and disappearance were not different from each other throughout the experiment (P>0.05).

NEFA concentration and turnover rate. Palmitate concentration, specific activity and fractional composition of total NEFA concentration measured during cold exposure experiments are presented in Figure 4.9. Concentration and isotopic steady-states were attained after 40 minutes of infusion (P>0.05). Palmitate concentration and fractional contribution to total NEFA averaged 0.18 ± 0.00 mM and 23.6 ± 0.1 %, respectively. While percentage of plasma palmitate remained constant (P=0.25), palmitate concentration increased 1.4 fold at the end of cold exposure (P<0.001). Similarly, palmitate specific activity increased significantly 30 minutes after reaching the lowest temperature (P<0.001). As shown in Figure 4.10, total NEFA concentration at steady-state was 0.76 ± 0.09 mM and increased significantly as temperature was lowered

Figure 4.8. Effect of cold exposure (6°C) on mean plasma glucose concentration (a), specific activity (b) and turnover rate (c) in 15°C acclimated rainbow trout. Gray zone indicates graded temperature decrease (n=8). * indicates a significant difference from steady-state values ($P<0.05$).

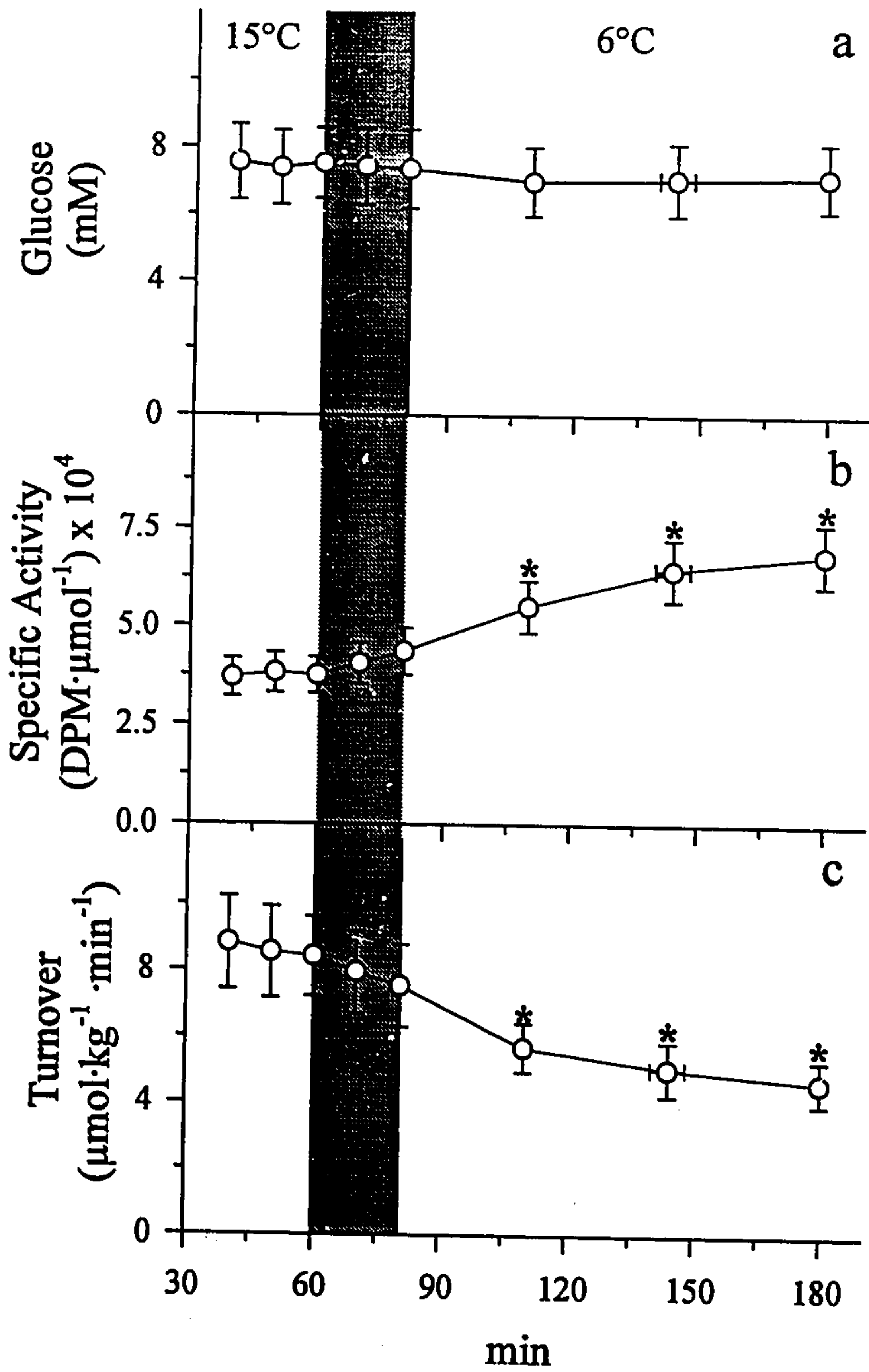

15°C
6°C
a
Glucose (mM)
8
4
0
b
Specific Activity (DPM·µmol-1) x 10^4
7.5
5.0
2.5
0.0
c
Turnover (µmol·kg-1·min-1)
8
4
0
30
60
90
120
150
180
min

Figure 4.9. Effect of cold exposure (6°C) on mean plasma palmitate concentration (a), specific activity (b) and fractional contribution to total NEFA concentration (c) in 15°C acclimated rainbow trout. Gray zone indicates graded temperature decrease (n=8). * indicates a significant difference from steady-state values (P<0.05).

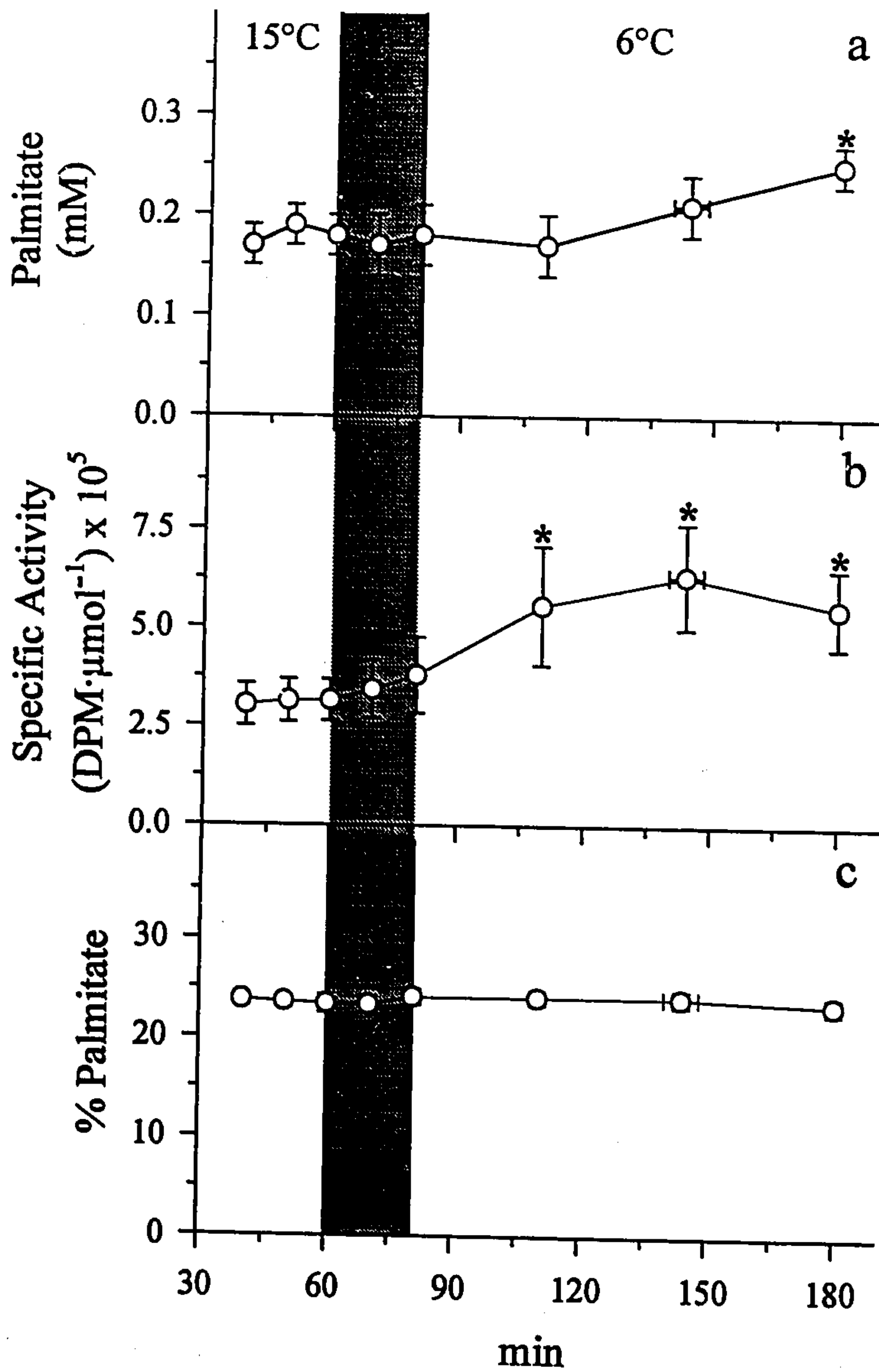
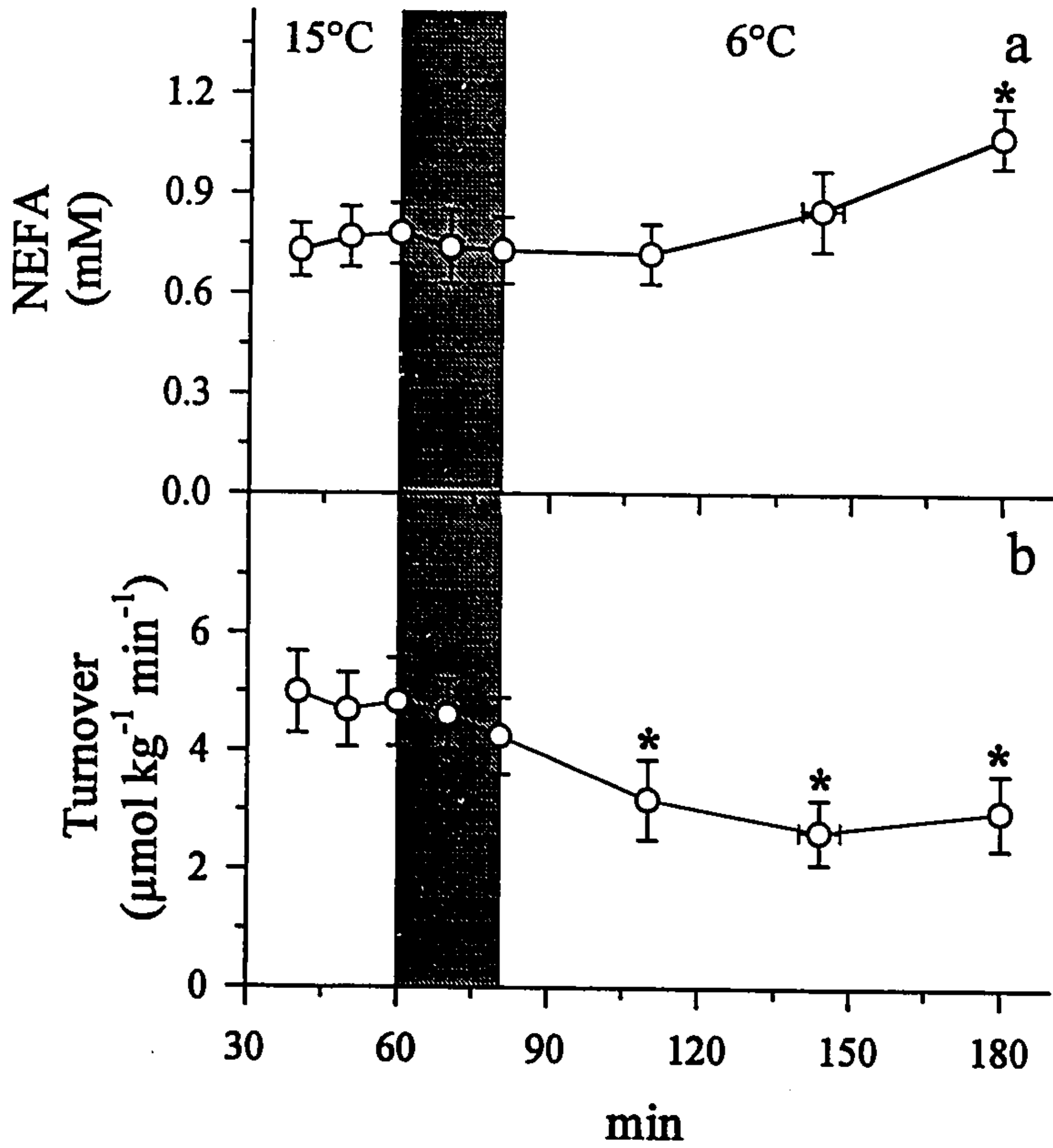

15°C
6°C
a
b
c
Palmitate (mM)
Specific Activity (DPM·µmol-1) x 10^5
% Palmitate
min
*

Figure 4.10. Effect of cold exposure (6°C) on mean plasma NEFA concentration (a) and turnover rate (b) in 15°C acclimated rainbow trout. Gray zone indicates graded temperature decrease (n=8). * indicates a significant difference from steady-state values ($P<0.05$).



15°C
6°C
a
b
NEFA
(mM)
1.2
0.9
0.6
0.3
0.0
Turnover
(μmol kg-1 min-1)
6
4
2
0
30
60
90
120
150
180
min

reaching a level 29% higher (1.07 ± 0.09 mM) at the end of the experiment ($P<0.0001$). When results for palmitate were extrapolated to total NEFA, steady-state NEFA turnover rate averaged 4.8 ± 0.7 μmol kg^{-1} min^{-1}. As temperature dropped by 9°C, NEFA turnover rate decreased to 3.2 ± 0.7 μmol $kg^{-1}min^{-1}$, 30 minutes after reaching 6°C and remained low until the end of the experiment ($P<0.001$) (Figure 4.10).

Effect of cold exposure on oxygen consumption and oxidative fuel fluxes

Changes in oxygen consumption, glucose and NEFA turnover rates during cold exposure are plotted as a percentage of control 15°C values (Figure 4.11). Glucose and NEFA fluxes responded similarly to 9°C drop in water temperature ($P>0.05$). In addition, Q_{10} values for oxygen consumption, glucose and NEFA turnover rates did not differ from each other (Table 4.4) ($P>0.05$).

Figure 4.11. Effect of cold exposure (6°C) on oxygen consumption (●), NEFA (○) and glucose (□) turnover rates in 15°C acclimated rainbow trout. Values presented as percent (% Rate) of 15°C control value (dotted line). Gray zone indicates graded temperature decrease (n=8). Glucose and NEFA fluxes were not different from each other ($P>0.05$).

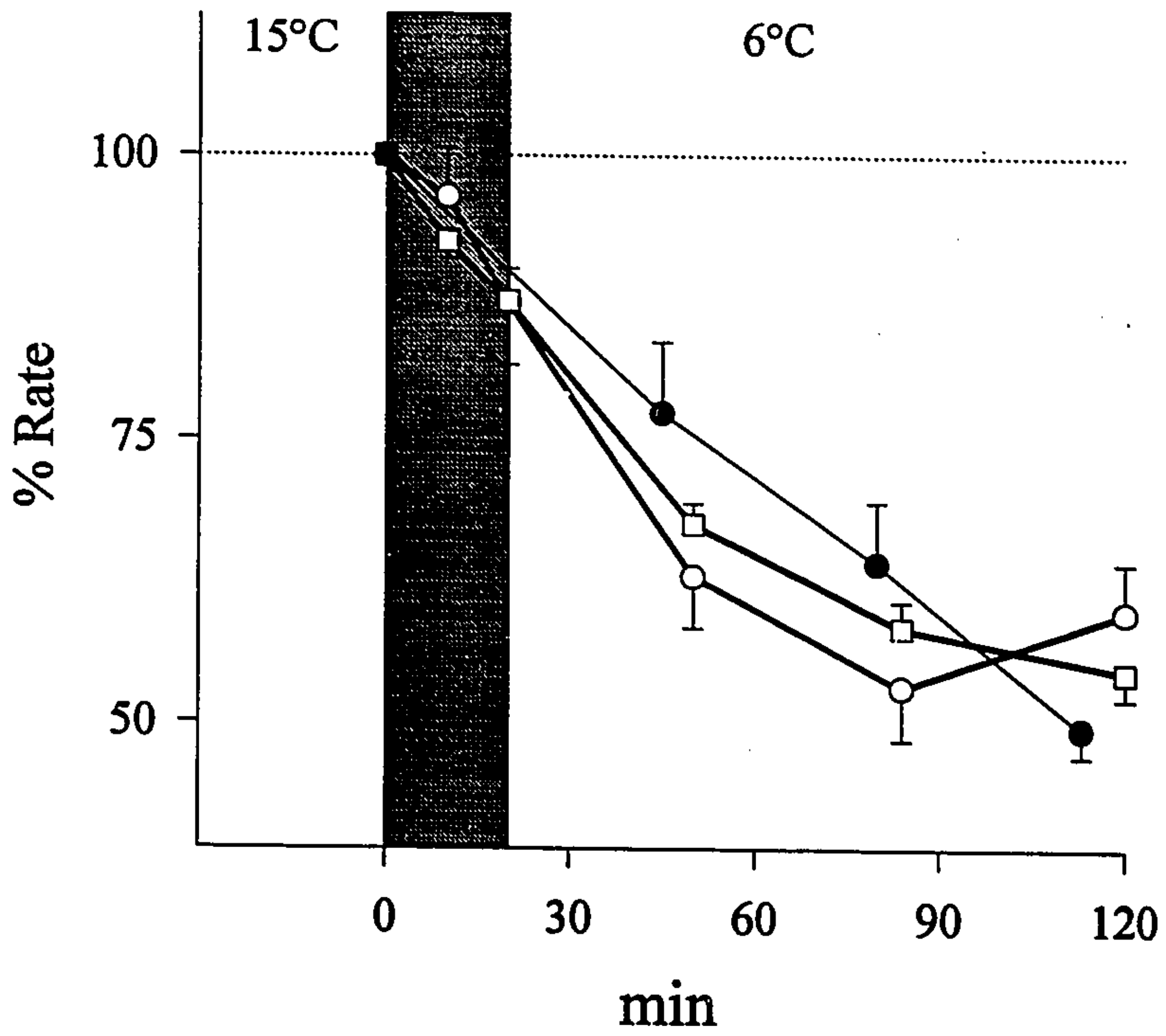

15°C
6°C
100
75
50
% Rate
0
30
60
90
120
min

Table 4.4. Average Q_{10} (15°C to 6°C) for oxygen consumption (MO_2), glucose (R_t Glucose) and NEFA (R_t NEFA) turnover rates during cold exposure.

	Q_{10}
MO_2	2.2 ± 0.2
R_t Glucose	1.9 ± 0.1
R_t NEFA	2.1 ± 0.1

DISCUSSION

This study provides the first *in vivo* measurements of glucose and NEFA fluxes for fish exposed to hypoxia and a rapid decrease in environmental temperature. I show that acute hypoxia causes a progressive decrease in NEFA turnover rate and a transient increase in hepatic glucose production. A 9°C drop in water temperature results in a parallel decrease in glucose and NEFA fluxes, following the change in metabolic rate. This study also demonstrates that changes in plasma glucose and NEFA concentrations do not necessarily reflect changes in glucose and NEFA fluxes.

EFFECT OF HYPOXIA

Oxygen consumption

Various researchers (Holeton and Randall, 1967; Hughes and Saunders, 1970; McKim and Goeden, 1982; Randall, 1982) have reported that trout do not conform to hypoxia by decreasing their metabolic rate. Here, oxygen consumption rate remained at normoxic levels even 90 minutes after reaching 40 torr (Figure 4.3). Hyperventilation, an energically expensive mechanism used to increase oxygen extraction capacity, was easily observed when fish were exposed to the hypoxic water.

Glucose metabolism

Glucose appearance rate was temporarily higher then its rate of disappearance early after reaching 25% oxygen saturation, resulting in a temporary hyperglycemia (Figure 4.4). In rainbow trout, as in other teleost, severe hypoxia was shown to cause the release of stress hormones which is often linked to hyperglycemia (Ince and Thorpe, 1977; van Raaij, 1994; White and Fletcher, 1989). Even though stress hormone levels were not measured here, the transient increase in hepatic glucose production may have been caused by an activation of glycogenolysis and gluconeogenesis through catecholamine stimulation as suggested by Wright *et al.* (1989). A constant increase in plasma lactate concentration was also observed after oxygen saturation reached 25%, indicating an activation of anaerobic glycolysis (Dunn and Hochachka, 1986; Dunn and Hochachka, 1987; Heath and Pritchard, 1965; Holeton and Randall, 1967; van Raaij, 1994). Anaerobic glycolysis seemed to be preferentially fueled by tissue glycogen reserves rather than circulatory glucose since glucose flux returned to normoxic levels following a transient increase in its rate of appearance. Dunn and Hochachka (1987) reported similar results after exposing rainbow trout to an oxygen tension of 30 torr for 3 h. In their study, plasma glucose concentration and turnover rate remained constant even if activation of anaerobic glycolysis was apparent throughout hypoxia. Unfortunately, these researchers used a bolus injection of radiolabeled isotope to estimate glucose turnover rate which only allowed one measurement of flux at normoxia and after 3h of hypoxia. Consequently, this technique could not detect the brief increase in glucose production I observed here.

NEFA metabolism

Previous work on the effects of hypoxia on NEFA metabolism yielded conflicting results with regards to changes in plasma concentrations. Plitsetskaya (1980) reported that some teleosts (e.g., trout, tench and lamprey) reacted to hypoxia by increasing NEFA concentrations, while in others (carp and goldfish) a reduction in plasma NEFA concentration was observed. More recently, Van Raaij (1994) showed that NEFA concentration decreased significantly in carp and decreased only briefly in trout before returning to normoxic levels. Here, plasma NEFA concentration remained constant throughout hypoxia while NEFA flux rates decreased progressively, reaching 53% of normoxic level by the end of the experiment (Figure 4.6). This change in NEFA flux indicates that the requirement for this metabolite as a fuel decreased during hypoxia. This drop in NEFA flux may be linked to the increase in plasma glucose concentration observed during hypoxia. Randle *et al.* (1963) suggested that hyperglycemia could inhibit NEFA mobilization in adipose tissue, independently of hormonal control. In humans, Carlson *et al.* (1991) reported that during pancreatic-pituitary clamps, hyperglycemia caused a 30% and 34% decrease in NEFA and glycerol turnover rates. This simultaneous decrease in NEFA and glycerol turnover rate suggested that hyperglycemia acts through the suppression of adipose tissue lipolysis and not by stimulation of FFA reesterification (Carlson *et al.*, 1991). Similar findings on the effect of glycaemia on NEFA mobilization have also been reported in other studies in humans (Wolfe and Peters, 1987) and in dogs (Park *et al.*, 1990). The exact mechanisms for this interaction are still unclear. Nevertheless, the decrease in NEFA flux observed here in trout, suggests that this

reciprocal interaction between glycaemia and NEFA mobilization may also be present in fish.

EFFECT OF COLD EXPOSURE

Ectotherms have virtually no biochemical and physiological defenses against changing environmental temperature (see Introduction). During acute thermal modification, the response is limited only to first line defenses such as the modulation of pre-existing enzymes (for review see Somero and Hochachka, 1984; Hazel and Prosser, 1974). Here, as a result of the sudden drop in temperature from 15°C to 6°C, glucose and NEFA fluxes decreased proportionally to oxygen consumption rate with a Q_{10} of approximately 2 (Table 4.4). Neither glucose nor NEFA were preferred because the fluxes of both oxidative fuels decreased similarly (Figure 4.11). Unlike glucose and NEFA fluxes, oxygen consumption did not plateau after trout was exposed to 6°C water for 90 minutes. However, the calculated Q_{10} value (2.0 ± 0.2) for oxygen consumption was equivalent to the reported acclimation Q_{10} value in rainbow trout for the same temperature range (Cameron, 1989; Rao, 1968). Reports in rainbow trout (Bullock, 1955) and in flounder (*Platichthys flesus*) (Duthie and Houlihan, 1982) suggest that their metabolism is affected very differently when temperature is raised than when it is lowered. In 15°C acclimated animals, both studies showed that there was an important overshoot in oxygen consumption compared to acclimated levels when temperature was rapidly raised by 10°C (5°C to 15°C) while the reverse change in temperature (15°C to 5°C) did not cause oxygen consumption values to drop under acclimation levels. This response to rapid temperature modification suggests

that fish are more tolerant to decreases than increases in water temperature (Duthie and Houlihan, 1982; Fry and Hochachka, 1970).

While plasma glucose concentration decreased overall by 0.5 mM during cold exposure, plasma NEFA concentration increased approximately 30% by the end of the experiment. Changes in concentration are caused by an imbalance between rates of appearance and disappearance of a metabolite. Because temperature has an important effect on reaction rates, these modifications in glucose and NEFA concentrations may indicate a differential impact of temperature on either reaction rates involved in their production or their uptake and utilization. Since changes in glucose and NEFA concentrations observed here are gradual, measurement of glucose and NEFA appearance and disappearance rates is not sensitive enough to clearly interpret differences between production and uptake. The impact of cold exposure on plasma NEFA concentration is much more important than for plasma glucose concentration. This observation may be the result of hormonal stimulation or be related to the very low solubility of NEFA in aqueous solutions and the requirement for lipid-binding proteins for their transport (Zwingelstein, personal communication).

Previous studies indicate that in fish many hormones cause the release NEFA release into plasma (for review see Sheridan 1988). These hormones are released under various conditions and their impact on NEFA mobilization might be observed on different time scales. Unfortunately, no information is available on hormone levels during acute cold exposure. Another possibility for the increased NEFA concentration could be related to lipid transport in plasma and cells. With the exception of very short chain fatty

acids (10C ≥), NEFA are essentially insoluble in aqueous solutions (Sheridan, 1988). When released in plasma, NEFAs are generally transported as a complex with lipid-binding proteins (i.e. lipoproteins: 90-95%; albumin-like proteins: 5-10%) (Hendersen, 1987). Thus, impact of cold exposure on protein structure might serve to explain the increase in plasma NEFA concentration observed during this study. Modification of the structure of plasma lipoproteins and albumin-like proteins could trap lipids, making their removal from plasma more difficult. In time, these lipids would accumulate in plasma causing an increase in total NEFA concentration. Finally, sudden changes in temperature compromise biomembrane fluidity and barrier properties (cellular and mitochondrial) thus affecting cell permeability and function (Hazel, 1973; Hendersen, 1987). Therefore, it is also possible that uptake of NEFAs by the utilizing tissues decreased due to modification in membrane fluidity with decreasing temperature. Increased rigidity of membranes could serve as a much better barrier for water insoluble molecules (i.e., lipids) than soluble ones (i.e., glucose).

Effect of hypoxia and cold exposure on plasma NEFA composition

In both experiments, fractional contribution of individual NEFA to total plasma NEFA concentration found in Tables 4.1 and 4.2 were similar to values reported by van Raaij *et al.* (1994) for rainbow trout. In addition, NEFA concentrations changed in parallel and fractional contribution of individual NEFA to total NEFA remained constant throughout hypoxia and cold exposure indicating that possibly no preferential mobilization or utilization is occurring. However, as mentioned in Chapter 1, we have to be extremely

careful when trying to draw conclusions about changes in flux using concentrations since they do not necessarily change in parallel. Mean coefficient of variation (CV) shows that palmitate (16:0) reflects closely changes in total NEFA concentration in rainbow trout during hypoxia and cold exposure (Table 4.3). Similar results were found in exercising dogs and goats, where McClelland *et al.* (1995) also found that 16:0 yielded the lowest CV of all short chain fatty acids involved in energy metabolism. Thus, they suggested that 16:0 would be the best choice of tracer in NEFA kinetics studies but argued that this may not be the case for all species. Here, 16:0 is very closely followed by 22:6 in both experiments. In fact, 22:6 has a lower coefficient of variance (CV) than 16:0 during hypoxia experiments. Since shorter chain fatty acids (C18 and less) are primarily used for energy metabolism (Weber and Zwingelstein, 1995), 16:0 also appears to be the most appropriate tracer to estimate NEFA flux in fish.

During cold exposure experiments, one technical difficulty arises when using 16:0 as a tracer to represent total NEFA flux. Polyunsaturated fatty acids (PUFA) play a primordial role in the reorganization of membrane phospholipids to maintain an appropriate fluidity during temperature alterations (Bell *et al.*, 1986; Hendersen, 1987). In fact, the substitution of phospholipid composition from saturated fatty acids to unsaturated fatty acids is an invariable response in fish exposed to colder water (reviewed by Hendersen, 1987 and Bell, 1986). Trout hepatocyte membranes were shown to be very dynamic structures being able to rapidly modify phospholipid composition in response to temperature change. Hence, when 20°C-acclimated trout hepatocytes are exposed to 6 hour at 5°C, membrane fluidity increases very rapidly reducing cold-induced rigidity by

50% (Williams and Hazel, 1994). This change in fluidity represented half of the compensation observed in membranes of fully acclimated organisms (Williams and Hazel, 1994). Williams and Hazel (1995) suggested that early alterations constitute emergency measures to contend with rapid temperature changes. Later on, more permanent, and perhaps more effective, changes will be introduced as the thermal perturbation continues (Williams and Hazel, 1995). Consequently, NEFA turnover rate measured using palmitate as a tracer might represent an underestimation of PUFA turnover rate due to their increased importance during temperature-induced membrane restructuring (Zwingelstein, personal communication).

CHAPTER 5

GENERAL CONCLUSION

The main purpose of my thesis was to measure, using a continuous infusion technique, changes in circulatory glucose and nonesterified fatty acids (NEFA) in rainbow trout exposed acutely to hypoxia and cold. The work was divided into three areas. First, the development of a surgical technique allowing placement of an infusing and a sampling catheter and adequate estimation of glucose flux (Chapter 2). Second, the accuracy of this continuous infusion method in estimating glucose turnover rate was verified (Chapter 3). Finally, this technique was used to measure the effect of two environmental perturbations on glucose and NEFA fluxes (Chapter 4). The following conclusions can be drawn from this thesis.

1. New cannulation technique and reliability of continuous infusion to estimate glucose flux *in vivo*:

The double dorsal aorta cannulation proposed is adequate to carry out simultaneous infusion of metabolic tracer and blood sampling. The surgical procedure is rapid, easy to perform and does not require extensive training. The continuous infusion technique also provides accurate estimates of glucose fluxes as demonstrated by experiments where normal glucose production was artificially replaced by a pump in hepatectomized fish. Resting glucose turnover rates estimated with this new and validated infusion method showed, that previous measurements made by bolus injection (a technique that has never been validated in fish) were strongly underestimating true fluxes.

2. Effects of hypoxia and cold exposure on glucose and NEFA fluxes:

Acute hypoxia causes a transient increase in the rate of appearance of glucose leading to short term hyperglycaemia. However, this flux returns to normoxic levels after 60 minutes of hypoxia suggesting that *in situ* utilization of tissue glycogen reserves rather than circulatory glucose is the major fuel for anaerobic glycolysis. Hypoxia also causes a 53% decrease in NEFA flux by the end of the experiment while having no effect on plasma NEFA concentration. This indicates that the requirement for NEFA as a metabolic fuel decreases during hypoxia.

Acute exposure to cold temperature causes glucose, NEFA and oxygen fluxes to decrease in parallel with similar Q_{10} of approximately 2. While glucose concentration only showed a slight overall decrease as a result of cold exposure, NEFA concentration increased by 30% after 100 minutes at 6°C. These changes in concentration are the result of small imbalances between the rates of appearance and disappearance of these metabolites.

3. Do metabolite flux and concentration change in parallel?

This thesis clearly shows that changes in plasma metabolite concentrations cannot be used to make inferences about changes in metabolite fluxes. Table 5.1 summarizes the effects of the two environmental stresses used here on concentration and fluxes of glucose and NEFA. It clearly shows that concentration and flux vary independently most of the time. Changes in concentrations merely reflect imbalances between rates of appearance and disappearance of the metabolite, and they indicate nothing about changes in flux. This further supports the importance of measuring metabolite fluxes directly when studying

Table 5.1. Overall changes in glucose and NEFA concentrations and fluxes during hypoxia and cold exposure experiments. Oxygen consumption (MO_2) is also indicated. Size of the arrow indicates relative magnitude of the change.

	Hypoxia		Cold exposure	
	[ ]	Flux	[ ]	Flux
Glucose	↑	—	↓ (small)	↓
NEFA	—	↓	↑	↓
MO_2	—		↓	

the impact of a perturbation on circulatory substrates. Future application of the continuous infusion technique under steady as well as non-steady state conditions should add a new dimension to the general understanding of fish metabolism.

REFERENCES

Allsop, J. R., R.R., W., DiStephano, J. J., and Burke, J. F. (1979). The reliability of rates of glucose appearance *in vivo* calculated from single tracer injections. *Can. J. Physiol. Pharmacol.*, *57*, 1267-1274.

Allsop, J. R., Wolfe, R. R., and Burke, J. F. (1978). The reliability of rates of glucose appearance in vivo calculated from constant tracer infusions. *Biochem. J.*, *172*, 407-416.

Andersen, D. E., Reid, S. D., Moon, T. W., and Perry, S. F. (1991). Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.*, *48*(9), 1811-1817.

Batty, R. S., and Wardle, C. S. (1979). Restoration of glycogen from lactic acid in the anaerobic swimming muscle of plaice *Pleuronectes platessa L. J. Fish Biol.*, *15*, 509-519.

Bell, M. V., Henderson, R. J., and Sargent, J. R. (1986). The role of polyunsaturated fatty acids in fish. *Comp. Biochem. Physiol.*, *83B*(4), 711-719.

Bergmeyer, H. U. (1985). *Methods of enzymatic analysis*. Weinheim: VCH.

Bever, K., Chenoweth, M., and Dunn, A. (1977). Glucose turnover in kelp bass (*Paralabrax sp.*): *in vivo* studies with (6-^{3}H, 6-^{14}C)glucose. *Am. J. Physiol.*, *232*, R66-R72.

Bever, K., Chenoweth, M., and Dunn, A. (1981). Amino acid gluconeogenesis and glucose turnover in kelp bass (*Paralabrax sp.*). *Am. J. Physiol.*, *240*, R246-R252.

Bilinski, E., and Jonas, R. E. E. (1972). Oxidation of lactate to carbon dioxide by rainbow trout (*Salmo gairdneri*) tissues. *J. Fish. Res. Board Can.*, *29*, 1467-1471.

Bolis, L., Luly, P., and Baroncelli, V. (1971). D(+)-Glucose permeability in brown trout *Salmo trutta* L. erythrocytes. *J. Fish. Biol.*, *3*, 273-275.

Boutilier, R. G., Dobson, G., Hoeger, U., and Randall, D. J. (1988). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairneri*): metabolic and respiratory adaptations. *Respira. Physiol.*, *71*, 69-82.

Bryan, J. D., Kelsch, S. W., and Neill, W. H. (1990). The maximum power principle in behavioral thermoregulation by fishes. *Transac. Am. Fish. Soc.*, *119*, 611-621.

Bullock, T. H. (1955). Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev.*, *30*, 311-342.

Cameron, J. N. (1989). *The repiratory physiology of animals*. New York: Oxford University Press.

Carlson, M. G., Snead, W. L., Hill, J. O., Nurjhan, N., and Campbell, P. J. (1991). Glucose regulation of lipid metabolism in humans. *Am. J. Physiol.*, *261*(24), E815-E820.

Carruthers, A. (1990). Facilitated diffusion of glucose. *Physiol. Rev.*, *70*(4), 1135-1176.

Cherry, D. S., Dickson, K. L., and Cairns Jr, J. (1975). Temperature selected and avoided by fish at various acclimation temperatures. *J. Fish. Res. Board Can.*, *32*, 485-491.

Clark, C. W., and Levy, D. A. (1988). Diel vertical migrations by juvenile sockeye salmon and the antipredation window. *Am. Naturalist*, *131*(2), 271-290.

Clarke, A. (1993). Temperature and extinction in the sea: a physiologist's view. *Paleobiology, 19*(4), 499-518.

Clarke, S. D., and Armstrong, M. K. (1989). Cellular lipid binding proteins: expression, function, and nutritional regulation. *FASEB J., 3,* 2480-2487.

Connor, W. E., Lin, D. S., and Colvis, C. (1996). Differential mobilization of fatty acids from adipose tissue. *J. Lipid Res., 37,* 290-298.

Cornish, I., and Moon, T. W. (1985). Glucose and lactate kinetics in American eel *Anguilla rostrata. Am. J. Physiol., 249,* R67-R72.

Cowey, C. B., de la Higuera, H. and, Adron, J. W (1977). The effect of dietary composition and of insulin on gluconeogenesis in rainbow trout *(Salmo gairdneri). Br. J. Nutr., 38,* 385-395.

Crawshaw, L. I., and Hazel, J. R. (1984). Temperature effect in fish. *Am. J. Physiol., 15,* R439-R440.

Davidson, W. S., Bartlett, S. E., Birt, T. P., Birt, V. L., and Green, J. M. (1989). Identification and purification of serum albumin from rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol., 93B*(1), 5-9.

Daw, J. C., Wenger, D. P., and Berne, R. M. (1967). Relationship between cardiac glycogen and tolerance to anoxia in the Western Painted Turtle (*Chrysemys picta bellii*). *Comp. Biochem. Physiol., 22,* 69-73.

Dunn, J. F., and Hochachka, P. W. (1986). Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. *J. Exp. Biol., 123,* 229-242.

Dunn, J. F., and Hochachka, P. W. (1987). Turnover rates of glucose and lactate in rainbow trout during acute hypoxia. *Can. J. Zool.*, *65*, 1144-1148.

Duthie, G. G., and Houlihan, D. F. (1982). The effect of single step and fluctuating temperature changes on the oxygen consumption of flounders, *Platichthys flesus* (L.): Lack of temperature adaptation. *J. Fish Biol.*, *21*, 215-226.

Felig, P., and Wahren, J. (1975). Fuel homeostasis in exercise. *New Engl. J. Med.*, *293*(21), 1078-1084.

Fellows, F. C. I., and Hird, F. J. R. (1981). Fatty acid binding proteins in the serum of various animals. *Comp. Biochem. Physiol.*, *68B*, 83-87.

Fritsche, R., and Nilsson, S. (1993). Cardiovascular and ventilatory control during hypoxia. In J. C. R. and. F. B. Jensen (Eds.), *Fish Ecophysiology* London: Chapman and Hall.

Fry, F. E. J. (1971). The effect of environmental factors on the physiology of fish. *In Fish Physiology*, Hoar and Randall (Eds.), Vol. VI (pp. 1-87). New York: Academic Press.

Fry, F. E. J., and Hochachka, P. W. (1970). Fish. In *Comparative physiology of thermoregulation,* G. C. Whittow (Eds.), (pp. 79-134). New York: Academic Press.

Garin, D., Rombaut, A., and Fréminet, A. (1987). Determination of glucose turnover in sea bass *Dicentrarchus labrax.* Comparative aspects of glucose utilization. *Comp. Biochem. Physiol.*, *87B*(4), 981-988.

Guderley, J. (1990). Functional significance of metabolic responses to thermal acclimation in fish muscle. *Am. J. Physiol.*, *259*(Regulatory integrative Comp. Physiol. 28), R245-R252.

Hagenfeldt, L. (1975). Turnover of individual free fatty acids in man. *Fed. Proc.*, *34*, 2236-2240.

Halver, J. E. (1972). *Fish Nutrition*. New York: Academic Press.

Hazel, J. R. (1973). The regulation of cellular function by temperature-induced alterations in membrane composition. In *Effects of Temperature on Ectothermic Organisms*, W. Wieser (Eds.), (pp. 55-67). New York: Springer-Verlag.

Hazel, J. R. (1984). Effects of temperature on the structure and metabolism of cell membranes in fish. *Am. J. Physiol.*, *246*(Regulatory Integrative Comp. Physiol. 15), R460-R470.

Hazel, J. R., and Prosser, C. L. (1974). Molecular mechanisms of temperature compensation in poikilotherms. *Physiol. Rev.*, *54*(3), 620-677.

Heath, A. G., and Pritchard, A. W. (1965). Effects of severe hypoxia on carbohydrate energy stores and metabolism in two species of fresh-water fish. *Physiol. Zool.*, *XXXVIII*(4), 325-334.

Hendersen, R. J. (1987). The lipid composition and biochemistry of freshwater fish. *Prog. Lipid Res.*, *26*, 281-347.

Hetenyi, G. J., Perez, G., and Vranic, M. (1983). Turnover and precursor-product relationships of non-lipid metabolites. *Physiol. Rev.*, *63*(2), 606-667.

Hochachka, P. W., and Guppy, M. (1987). *Metabolic Arrest and the Control of Biological Time*. Cambridge: Harvard University Press.

Hochachka, P. W., and Somero, G. N. (1984). *Biochemical Adaptation*. Princeton, NJ: Princeton University Press.

Holeton, G. F., and Randall, D. J. (1967). The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. *J. Exp. Biol.*, *46*, 317-327.

Hughes, G. M., and Saunders, R. L. (1970). Responses of the respiratory pumps to hypoxia in the rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.*, *53*, 529-545.

Ince, B. W., and Thorpe, A. (1977). Plasma insulin and glucose responses to glucagon and catecholamines in the European silver eel (*Anguilla anguilla* L.). *Gen. Comp. Endocrino.*, *33*, 453-459.

Johnston, I. A. (1975). Studies on the swimming musculature of the rainbow trout. II. Muscle metabolism during severe hypoxia. *J. Fish Biol.*, *7*, 459-467.

Katz, J. (1979). Use of isotopes for the study of glucose metabolism *in vivo*. *Techniques in metabolic reasearch, B207*, 1-22.

Katz, J. (1992). On the determination of turnover in vivo with tracers. *Am. J. Physiol.*, *263*(26), E417-E424.

Katz, J., Dunn, A., Chenoweth, M., and Golden, S. (1974). Determination of synthesis, recycling, and body mass of glucose in rats and rabbits *in vivo* with ^{3}H- and ^{14}C-labelled glucose. *Biochem. J.*, *142*, 171-183.

Kelsch, S. W., and Neill, W. H. (1990). Temperature preference versus acclimation in fishes: selection for changing metabolic optima. *Transac. Am. Fish. Soc., 119*, 601-610.

Kerem, D., Hammond, D., and Elsner, R. (1973). Tissue glycogen levels in the Weddell seal *Leptonychotes weddelli*: A possible adaptation to asphyxial hypoxia. *Comp. Biochem. Physiol., 45A*, 731-736.

Knox, D., Walton, M. J., and Cowey, C. B. (1980). Distribution of enzymes of glycolysis and gluconeogenesis in fish tissues. *Mar. Biol., 56*, 7-10.

Levy, J. C., Brown, G., Matthews, D. R., and Turner, R. C. (1989). Hepatic glucose output measured with labeled glucose to reduce negative errors. *Am. J. Physiol., 257*(20), E531-E540.

Lin, H., Romsos, D. R., Tack, P. I., and Leveillé, G. A. (1978). Determination of glucose utilization in coho salmon (*Oncorhynchus kisutch*, Walbaum) with (6-^{3}H) and (U-^{14}C) glucose. *Comp. Biochem. Physiol., 59A*, 189-191.

Machado, C. R., Garofalo, M. A. R., Roselino, J. E. S., Kettlehut, I. C., and Migliorini, R. H. (1989). Effect of fasting on glucose turnover in a carnivorous fish (*Hoplias* sp.). *Am. J. Physiol., 256*, R612-R615.

Maillou, J., and Nimmo, I. A. (1993). Albumin-like proteins in the serum of rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol., 104B*(2), 387-393.

Marsh, R. L., and Dawson, W. R. (1982). Substrate metabolism in seasonally acclimatized American goldfinches. *Am. J. Physiol., 242*, R563-R569.

McClelland, G., Zwingelstein, G., Taylor, C. R., and Weber, J.-M. (1995). Effects of exercise on the plasma non-esterified fatty acid composition of dogs and goats: species with different aerobic capacities and diets. *Lipids*, *30*(2), 147-153.

McKim, J. M., and Goeden, H. M. (1982). A direct measure of the uptake efficiency of a xenobiotic chemical accross the gills of brook trout (*Salvelinus fontinalis*) under normoxic and hypoxic conditions. *Comp. Biochem. Physiol.*, *72C*(1), 65-74.

Moates, J. M., Lacy, D. B., Goldstein, R. E., Cherrington, A. D., and Wasserman, D. H. (1988). Metabolic role of the exercise-induced increment in epinephrine in the dog. *Am. J. Physiol.*, *255*(18), E428-E436.

Molina, J. M., Baron, A. D., Edelman, S. V., Brechtel, G., Wallace, P., and Olefsky, J. M. (1990). Use of variable tracer infusion method to determine glucose turnover in humans. *Am. J. Physiol.*, *258*(21), E16-E23.

Mommsen, T. P., and Moon, T. W. (1990). Metabolic response of teleost hepatocytes to glucagon-like peptide and glucagon. *J. Endocrino.*, *126*, 109-118.

Norwich, K. H. (1992). Sites of infusion and sampling for measurement of rates of production in steady state. *Am. J. Physiol.*, *263*(26), E817-E822.

Park, K. S., Rhee, B. D., Lee, K. U., Lee, H. K., Koh, C.-S., and Min, H. K. (1990). Hyperglycemia per se can reduce plasma free fatty acid and glycerol levels in the acutely insulin-deficient dog. *Metab. Clin. Exp.*, *39*, 595-597.

Perry, S. F., and Wood, C. M. (1989). Control and coordination of gas transfer in fishes. *Can. J. Zool.*, *67*, 2961-2970.

Pesquero, J., Roig, T., Bermudez, J., and Sanchez, J. (1994). Energy metabolism by trout red blood cells: substrate utilisation. *J. exp. Biol.*, 183-190.

Peters Jr, T., and Davidson, L. K. (1991). Isolation and properties of a fatty acid-binding protein from the Pacific lamprey (*Lampetra tridentata). Comp. Biochem. Physiol.*, *99B*(3), 619-623.

Plitsetskaya, E. (1980). Fatty acid levels in blood of cyclostomes and fish. *Env. Biol. Fish.*, *5*, 273-290.

Randall, D. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol.*, *100*, 275-288.

Randall, D. J., and Perry, S. F. (1992). Catecholamines. In *The Cadiovascular System*, W. S. Hoar, D. J. Randall, and A. P. Farrel (Eds.), (pp. 255-300). New York: Academic Press.

Randle, P. J., Garland, P. B., Hales, C. N., and Newsholme, E. A. (1963). The glucose-fatty acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet 1*, 785-789.

Rao, G. M. M. (1968). Oxygen consumption of rainbow trout (*Salmo gairdneri*) in relation to activity and salinity. *Can. J. Zool.*, *46*, 781-786.

Sephton, D. H., and Driedzic, W. R. (1994). Glucose metabolism by sea raven (*Hemitripterus Americanus*) and rainbow trout (*Oncorhynchus mykiss*) erythrocytes. *J. exp. Biol.*, *194*, 167-180.

Sheridan, M. A. (1988). Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. *Comp. Biochem. Physiol.*, *90B*, 679-690.

Sidell, B. D., and Hazel, J. R. (1984). Temperature effects on diffusivities of metabolites through cytosol of fish muscle. *Am. Zool.*, *24*, 96A.

Sidell, B. D., and Hazel, J. R. (1987). Temperature affects the diffusion of small molecules through cytosol of fish muscle. *J. Exp. Biol.*, *129*, 191-203.

Smith, L. S., and Bell, G. R. (1964). A technique for prolonged blood sampling in free-swimming salmon. *J. Fish. Res. Board Can.*, *21*(4), 711-717.

Smith, U. (1983). Adrenergic control of lipid metabolism. *Acta Med. Scand. suppl.*, *672*, 41-47.

Soivio, A., Nyholm, K., and Westman, K. (1975). A technique for repeated sampling of the blood of individual resting fish. *J. Exp. Biol.*, *62*, 207-217.

Sokoloff, L. (1983). The (^{14}C) deoxyglucose method for the quantitative determination of local cerebral glucose utilization: theoretical and practical considerations. In *Cerebral Metabolism and Neural Function*, J. V. Passonneau, R. A. Hawkins, W. D. Lust, and F. A. Welsh (Eds.), (pp. 319-330). Baltimore: Williams and Wilkins.

Spitzer, J. J. (1975). Application of tracers in studying free fatty acid metabolism of various organs in vivo. *Fed. Proc.*, *34*(13), 2242-2245.

Steele, R. (1959). Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann. NY Acad. Med. Sci.*, *82*, 420-430.

Steffensen, J. F. (1989). Some errors in respirometry of aquatic breathers: how to avoid and correct for them. *Fish. Physiol. and Biochem.*, *6*(1), 49-59.

Stewart, J. M., and Driedzic, W. R. (1988). Fatty acid binding proteins in teleost fish. *Can. J. Zool.*, *66*, 2671-2675.

Suarez, R. K., and Mommsen, T. P. (1987). Gluconeogenesis in teleost fish. *Can. J. Zool.*, *65*, 1869-1882.

Sweetser, D. A., Heuckeroth, R. O., and Gordon, J. I. (1987). The metabolic significance of mammalian fatty-acid-binding proteins: Abundant proteins in search of a function. *Ann. Rev. Nutr.*, *7*, 337-359.

Tse, C.-M., and Young, J. D. (1990). Glucose transport in fish erythrocytes: variable cytochalasin-B-sensitive hexose transport activity in the common eel (*Anguilla japonica*) and transport deficiency in the paddyfield eel (*Monopterus albus*) and rainbow trout (*Salmo gairdneri*). *J. exp. Biol.*, *148*, 367-383.

Tserng, K.-Y., Kliegman, R. M., Miettinen, E.-L., and Kalham, S. C. (1981). A rapid, simple, and sensitive procedure for the determination of free fatty acids in plasma using glass capillary column liquid chromatography. *J. Lipid Res.*, *22*, 852-858.

van den Thillart, G. (1982). Adaptations of fish energy metabolism to hypoxia and anoxia. *Mol. Physiol.*, *2*, 49-61.

van den Thillart, G. (1986). Energy metabolism of swimming trout (*Salmo gairdneri*). *J. Comp. Physiol.*, *156*, 511-520.

Van Ginneken, V., van den Thillart, G., Addink, A., and Erkelens, C. (1995). Fish muscle energy metabolism measured during hypoxia and recovery: an in vivo ^{32}P-NMR study. *Am. J. Physiol.*, *268*, R1178-R1187.

van Raaij, M. (1994) *Lipid metabolism in teleost fish and the hormonal control of substrate mobilization during hypoxia and anoxia*. Ph.D. thesis, Rijkuniversiteit te Leiben.

Vijayan, M. M., and Moon, T. W. (1994). The stress response and the plasma disappearance of corticosteroid and glucose in a marine teleost, the sea raven. *Can. J. Zool.*, *72*, 379-386.

Washburn, B. S., Bruss, M. L., Avery, E. H., and Freedland, R. A. (1992). Effects of estrogen on whole animal and tissue glucose use in female and male rainbow trout. *Am. J. Physiol.*, *32*, R1241-R1247.

Weber, J.-M., Brill, R. W., and Hochachka, P. W. (1986). Mammalian metabolite flux rates in a teleost: lactate and glucose turnover in tuna. *Am. J. Physiol.*, *250*, R452-R458.

Weber, J.-M., and Haman, F. (1996). Pathways for metabolic fuels and oxygen in high performance fish. *Comp. Biochem. Physiol.*, *113A*(1), 33-38.

Weber, J.-M., and Zwingelstein, G. (1995). Circulatory substrate fluxes and their regulation. In *Biochemistry and Molecular Biology of Fishes*, P.W. Hochachka and T. P. Mommsen (Eds.), Vol. 4, (pp.15-32), Elsevier Science.

West, T. G., Arthur, P. G., Suarez, R. K., Doll, C. J., and Hochachka, P. W. (1993). *In vivo* utilization of glucose by heart and locomotory muscles of exercising rainbow trout (*Onchorhynchus mykiss*). *J. Exp. Biol.*, *177*, 63-79.

West, T. G., Brauner, C. J., and Hochachka, P. W. (1994a). Muscle glucose utilization during sustained swimming in the carp (*Cyprinus carpio*). *A. J. Physiol.*, *267*(36), R1226-R1234.

West, T. G., Schulte, P. S., and Hochachka, P. W. (1994b). Implication of hyperglycemia for post-exercise resynthesis of glycogen in trout skeletal muscle. *J. exp. Biol.*, *189*, 69-84.

White, A., and Fletcher, T. C. (1989). The effect of physical disturbance, hypoxia and stress hormones on serum components of the plaice, *Pleuronectes platessa* L. *Comp. Biochem. Physiol.*, *93A*(2), 455-461.

Williams, E. E., and Hazel, J. R. (1994). Membrane fluidity and hemilayer temperature sensitivity in trout hepatocytes during brief *in vitro* cold exposure. *Am. J. Physiol.*, *266*(Regulatory Integrative Comp. Physiol. 35), R773-R780.

Williams, E. E., and Hazel, J. R. (1995). Restructuring of plasma membrane phospholipids in isolated hepatocytes of rainbow trout during brief *in vitro* cold exposure. *J. Comp. Physiol.*, *B*, 600-608.

Wojcieszyn, J. W., Schlegel, R. A., Wu, E.-S., and Jacobson, K. A. (1981). Diffusion of injected macromolecules within cytoplasm of living cells. *Proc. Natl. Acad. Sci. USA*, *78*(7), 4407-4410.

Wolf, K. (1963). Physiological saline for fresh water teleosts. *Prog. Fish Cult.*, *25*, 135-140.

Wolfe, R. R. (1992). *Radioactive and Stable Isotope Tracers in Biomedecine. Principles and Practice of Kinetic Analysis*. New York: Wiley-Liss.

Wolfe, R. R., and Peters, E. J. (1987). Lipolytic response to glucose infusion in human subjects. *Am. J. Physiol.*, *252*(15), E218-E223.

Wright, P. A., Perry, S. F., and Moon, T. W. (1989). Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J. exp. Biol.*, *147*, 169-188.