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**THE EFFECTS OF PROLONGED FASTING, HIBERNATION AND  
LEPTIN ON *IN VIVO* LIPID METABOLISM IN MAMMALS.**

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## **ABSTRACT**

The main purpose of this thesis was to identify the changes that occur in *in vivo* mammalian lipid metabolism during long-term fasting, arousal from hibernation, and following leptin treatment. Rabbits (*Oryctolagus cuniculus*) and woodchucks (*Marmota monax*) were fasted for one and two weeks, respectively, at room temperature and during different seasons. Woodchucks fasted in the summer lowered their energy expenditure and protein oxidation as soon as the fast was initiated. The woodchucks fasting in the spring did not, as their metabolic rate and protein utilization were already low, probably because in the wild they fast for extended periods of time following their final arousal from hibernation in the spring. The rabbits also exhibited metabolic depression and protein sparing with fasting, although they used more protein and less lipid than the woodchucks.

Using the continuous infusion method, rates of triacylglycerol (TAG) hydrolysis (lipolysis), fatty acid (FA) mobilization and FA reesterification were measured on woodchucks that had just aroused from three months of hibernation. The contribution of TAG/FA substrate cycling to their total energy expenditure was extremely high (17%). Also, thermogenesis by this substrate cycle decreased the longer an animal had been aroused from hibernation, suggesting that this substrate cycle may be an important source of heat during arousal. Due to the high TAG/FA cycling rates, their lipolytic rate was also extremely high.

Using the same methods, temporal changes in the rates of FA reesterification, lipolysis and lipid mobilization were measured in fasting rabbits. Their rates of lipolysis and FA mobilization initially doubled, however, as the fast was prolonged, those rates decreased to pre-fasting levels. The metabolic depression induced by fasting probably

accounted for the changes as less substrate was required for  $\beta$ -oxidation. Reduced TAG/FA substrate cycling with long-term fasting accounted for a small amount of this metabolic depression (3%).

The effects of leptin on TAG/FA recycling, lipolysis and FA mobilization were also investigated. *In vivo* leptin treatment caused significant increases in lipolytic rates and FA mobilization, and increased the relative contribution of lipid to the rabbit's total energy expenditure. The cycling rate of the TAG/FA substrate cycle was stimulated by leptin, contributing to 15% of the leptin-induced increase in energy expenditure. This showed that substrate cycling activation is a potent mechanism by which leptin can increase metabolic thermogenesis.

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Le but principal de cette thèse était d'identifier les changements du métabolisme lipidique des mammifères prenant place *in vivo* pendant un jeûne prolongé et au cours du réveil de l'hibernation, ainsi que d'étudier le rôle de la leptine dans la régulation de ces processus métaboliques.

Des lapins (*Oryctolagus cuniculus*) et des marmottes (*Marmota monax*) ont subi un jeûne d'une ou deux semaines, respectivement, à 22°C et à des saisons différents. Les marmottes jeûnant pendant l'été abaissèrent leur dépense énergétique et leur taux

d'oxydation des protéines aussitôt qu'elles arrêtaient de manger. Les marmottes jeûnant au printemps n'eurent pas cette réponse, leur taux métabolique et d'utilisation des protéines étant déjà bas, probablement parce que, à l'état sauvage, elles jeûnent pour de longues périodes à la suite de leur réveil printanier de l'hibernation. Les lapins démontrèrent aussi une dépression métabolique et une économie des protéines pendant le jeûne, bien qu'ils utilisèrent plus de protéines et moins de lipides que les marmottes.

Les taux d'hydrolyse du triacylglycerol (TAG) ou de lipolyse, de mobilisation des acides gras libres (AGL) et de réesterification des acides gras ont été mesurés par la méthode d'infusion continue sur des marmottes qui venaient de se réveiller après 3 mois d'hibernation. La contribution relative du cycle de substrat TAG/AGL à la dépense totale d'énergie était extrêmement élevée (17%). Aussi, la thermogenèse occasionée par ce cycle diminua progressivement après le réveil de l'hibernation, ce qui suggère que ce cycle est peut-être une source importante de chaleur pendant le réveil. Le taux de lipolyse était aussi extrêmement élevée pour pouvoir supporter ce cycle très actif.

En utilisant les mêmes techniques d'infusion, les changements temporeux des taux de réesterification des AGL, de lipolyse, et de mobilisation des lipides ont été mesurés sur des lapins pendant le jeûne. Leurs taux de lipolyse et de mobilisation des AGL doublèrent initialement, cependant, alors que le jeûne se prolongeait, ces taux retournèrent à leur niveau d'avant le jeûne. La dépression métabolique produite par le jeûne est probablement responsable de ces changements à long terme, puisque moins de substrat devient nécessaire pour la  $\beta$ -oxydation. La réduction de flux à travers le cycle TAG/AGL au cours du jeûne prolongé ne comptait que pour une part faible de la dépression métabolique (3%).

Les effets de la leptine sur le cycle TAG/AGL, la lipolyse, et la mobilisation des AGL ont aussi été étudiés. Le traitement à la leptine *in vivo* causa une augmentation significative du taux de lipolyse, de la mobilisation des AGL, et augmenta la contribution relative des lipides à la dépense énergétique totale des lapins. Le taux de recyclage TAG/AGL fut aussi stimulé par la leptine, et cette réponse contribua à 15% de l'augmentation du taux métabolique provoqué par la leptine. Ceci démontre que l'activation des cycles de substrats est un mécanisme puissant pour augmenter la thermogénèse métabolique.

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## **LIST OF ABBREVIATIONS**

<b>ATP</b>	<b>adenosine triphosphate</b>	<b>TAG</b>	<b>triacylglycerol</b>
<b>C</b>	<b>carbon</b>	<b>TLC</b>	<b>thin layer chromatography</b>
<b>cAMP</b>	<b>cyclic adenosine monophosphate</b>	<b>UCP</b>	<b>uncoupling protein</b>
<b>CAT</b>	<b>catecholamine</b>		
<b>CoA</b>	<b>coenzyme A</b>		
<b>DPM</b>	<b>disintegration per minute</b>		
<b>FA</b>	<b>fatty acid</b>		
<b>GC</b>	<b>gas chromatography</b>		
<b>H</b>	<b>hydrogen</b>		
<b>J</b>	<b>joule</b>		
<b>LBM</b>	<b>lean body mass</b>		
<b>mRNA</b>	<b>messenger ribonucleic acid</b>		
<b>N</b>	<b>nitrogen</b>		
<b>NEFA</b>	<b>non-esterified fatty acid</b>		
<b>O<sub>2</sub></b>	<b>oxygen</b>		
<b>PE</b>	<b>polyethylene</b>		
<b>Ra</b>	<b>rate of appearance</b>		
<b>SA</b>	<b>specific activity</b>		
<b>STAT</b>	<b>signal transduction and activators of transcription</b>		
<b>T<sub>3</sub></b>	<b>triiodothyronine</b>		
<b>T<sub>4</sub></b>	<b>thyroxine</b>		

## **CHAPTER 1**

### **GENERAL INTRODUCTION**

### *Lipid storage and mobilization*

Food enters the body as a mixture of lipids, carbohydrates and protein which are either used immediately or stored as a future source of energy as triacylglycerol and glycogen. Most of this energy is stored in adipocytes as triacylglycerol, while the glycogen that can be stored in muscle and liver is small. Therefore, when an animal needs to rely on endogenous fuel for any extended length of time lipids become the primary energy source, with some additional energy being mobilized from protein as amino acids. Before energy can be obtained from triacylglycerol it must be hydrolyzed into its components, three fatty acids and one glycerol. Incomplete hydrolysis of triacylglycerol into mono- and diacylglycerol is negligible (Arner and Ostman, 1974). Triacylglycerol hydrolysis, or lipolysis, is catalyzed by hormone sensitive lipase. Catecholamines and glucagon activate this enzyme through the cyclic AMP (cAMP) pathway. Therefore, cAMP is a regulator of lipolysis because it controls cellular concentrations of the active form of hormone sensitive lipase. Low insulin levels also act to stimulate lipolysis because the inhibitory effects of insulin on cAMP levels are lowered. This disinhibition is critical for the increase in lipolysis that occurs with fasting. The other major hormone that recently has been recognized as a regulator of lipolysis is leptin (see review by Reidy and Weber, 2000).

Fatty acids released by lipolysis have two possible fates:  $\beta$ -oxidation to produce ATP or reesterification back into triacylglycerol. Lipolysis of muscle and liver cell triacylglycerol produce fatty acids that can be oxidized within those cells, while fatty acids released from adipocyte triacylglycerol must be released into the blood, bound to albumin and circulated to other cells because adipocytes have low oxidizing capabilities. Even if

there is not an oxidative demand for fatty acids, lipolysis is a continuous process in adipocytes. In fact, most hydrolyzed fatty acids are actually reesterified back into triacylglycerol and not oxidized. There is an absence of glycerol kinase in adipocytes, therefore, glycerol 3-phosphate made from glucose is used for this reesterification rather than the glycerol released from triacylglycerol (Newsholme and Taylor, 1969). This also means that all the glycerol released by lipolysis in adipose tissue will appear in the plasma for transport to other tissues. Fatty acid reesterification can occur both in the cell where lipolysis occurred (primary reesterification), and in a different cell if the fatty acid moves into the circulation and enters another cell (secondary reesterification) (Campbell *et al.*, 1992; Wolfe *et al.*, 1990). This cycle of triacylglycerol hydrolysis into fatty acids and glycerol and then reesterification back into triacylglycerol with a 'new' glycerol is called the triacylglycerol/fatty acid (TAG/FA) substrate cycle (Newsholme and Crabtree, 1976).

### *Substrate cycles*

A substrate cycle exists when concurrent flux (rate of appearance;  $R_a$ ) through opposing reactions catalyzed by different non-equilibrium enzymes use energy without net conversion of substrate to product. Such cycles consume energy and produce heat yet do not result in an increase in the production of an end product and, therefore, are sometimes called 'futile' cycles. Newsholme and Crabtree (1976) showed that these cycles can amplify the control of substrate flux in response to a given change in its regulator (*e.g.* substrate or a hormone). For example, the TAG/FA cycle increases the sensitivity of epinephrine control on fatty acid flux by up to 50% (Miyoshi *et al.*, 1988). This amplification also ensures that if there is a sudden increase or decrease in energy

expenditure and substrate requirements for oxidation it will not result in dangerous fluctuations in circulatory substrate concentrations (*e.g.* fatty acids) (Bahr *et al.*, 1990; Wolfe *et al.*, 1990).

The production of heat without net formation of substrate makes these cycles a potentially potent source of metabolic heat (Newsholme, 1978; Newsholme and Crabtree, 1976). The cycling rate of the TAG/FA and the fructose 6-phosphate/fructose bisphosphate substrate cycle both contribute to the thermogenic capacity of a variety of different animals (Clark *et al.*, 1973a; Clark *et al.*, 1973b; Newsholme and Crabtree, 1976; Wolfe *et al.*, 1987a). Studies on humans also show that these substrate cycles may actually play a role in regulation of energy expenditure and storage, essentially helping to determine the metabolic efficiency of individuals, and maybe, their tendency to become obese. For example, adipocytes of obese or previously obese humans have been shown to generate less heat than adipocytes of lean humans (Nilsson-Ehle and Nordin, 1985; Sorbris *et al.*, 1982), and the *in vivo* TAG/FA substrate cycling rates of humans as well as their isolated adipocytes has been shown to be negatively correlated with obesity (Bottcher and Furst, 1997; Wolfe *et al.*, 1987b).

### *Leptin*

Leptin is a hormone that is primarily produced by adipose tissue and the rate of leptin secretion and its plasma concentration are correlated with total fat mass (Hamilton *et al.*, 1995; Klein *et al.*, 1996; Maffei *et al.*, 1995). Therefore, this hormone circulates as an internal signal indicating the size of body fat stores. Its amino acid sequence exhibits high homology and has been determined for several mammals (Trudeau, 1998) and

possibly poultry (Friedman-Einat *et al.*, 1999; Taouis *et al.*, 1998). To date, the leptin protein has not been identified in lizards, amphibians, insects or fish. However, it is too early to state whether this is an indication that leptin is a protein found only in endothermic animals. There are at least 6 isoforms of the leptin receptor (Ob-R) (Lee *et al.*, 1996) and they are variably found in the hypothalamus, cerebral cortex, cerebellum, choroid plexus, lung, kidney, skeletal muscle, liver, pancreas, adipose tissue and adrenal medulla (Cao *et al.*, 1997; Golden *et al.*, 1997; Kieffer *et al.*, 1996; Lee *et al.*, 1996; Tartaglia *et al.*, 1995). Leptin is an important regulator of puberty, reproduction, thermoregulation, appetite, weight regulation and metabolic function (Cunningham *et al.*, 1999; Rosenbaum and Leibel, 1998; Halaas *et al.*, 1995; Pellymouner *et al.*, 1995; Reidy and Weber, 2000).

Considerable information has been gained about the various physiological functions of leptin by examining differences between wild-type rodents and their counterparts with single gene mutations causing either the suppression of normal leptin production or the expression of dysfunctional leptin receptors. These mutant animals exhibit severe obesity due to hyperphagia, hypometabolism and preferential fat storage, and are usually insulin resistant. Subsequently, these animals have been used for years as models of obesity and non-insulin-dependent diabetes mellitus (Type 2 diabetes). In *ob/ob* mice, the gene coding for leptin is mutated and produces a form of the hormone that is prematurely terminated and, therefore, cannot be recognized by the receptors (Zhang *et al.*, 1994). Obese *db/db* mice have a point mutation in the gene that specifically transcribes for the hypothalamic long isoform of the leptin receptor resulting in suppressed expression of this isoform and increased expression of a much shorter isoform that is

unable to activate the proteins responsible for Signal Transduction and Activation of Transcription (STAT proteins) (Ghilardi *et al.*, 1996; Lee *et al.*, 1996; Tartaglia *et al.*, 1995). Zucker *fa/fa* rats also have a dysfunctional leptin receptor. An amino acid substitution in the extracellular domain of the protein caused by a missense mutation in the OB-R gene results in impaired activation of some STAT proteins (Phillips *et al.*, 1996; White *et al.*, 1997). Therefore, the abnormally large fat reserves of *db/db* mice and *fa/fa* rats are the consequence of dysfunctional receptors; they are not caused by leptin itself because circulating levels of the hormone are very high in both animal models.

A number of experiments have shown that leptin has a direct autocrine or paracrine mode of action on the rates of synthesis and degradation of lipids (Bai *et al.*, 1996; Frühbeck *et al.*, 1998; Frühbeck *et al.*, 1997; Muoio *et al.*, 1997; Sarmiento *et al.*, 1997; Shimabukuro *et al.*, 1997). However, these experiments have primarily used *in vitro* cell or tissue preparations while none have looked at the *in vivo* effects of leptin on rates of lipolysis and fatty acid mobilization. Therefore, the effects of leptin on lipid metabolism *in vivo*, where many other regulating factors exist, is still speculative.

### *Fasting*

There are three key mechanisms used by mammals to survive prolonged periods of fasting. The first is for the animal to preferentially utilise fat over protein. Glycogen stores in a fasting animal are depleted very quickly so energy derived from this source is negligible. The use of fat as the primary fuel during a fast makes bioenergetic sense for a number of reasons. One gram of lipid gives approximately twice as much energy as one gram of protein or glucose (9 kcal g<sup>-1</sup> vs. 4 kcal g<sup>-1</sup>), lipids can be stored lighter than

proteins or glycogen and, finally, lipids are a true stored fuel, unlike proteins that are necessary for other body functions (cardiac and skeletal muscle, enzymes, etc.). This final reason is why sparing of body proteins while fasting is so important. When too much cardiac muscle has been degraded due to excessive protein loss, the heart can no longer function properly leading to death (Garnett *et al.*, 1969; Van Itallie and Yang, 1984). Therefore, all mammals, even those that do not go through annual periods of fasting-feeding cycles, utilise fat preferentially and conserve protein when fasted.

The second mechanism used by mammals to prolong survival during a fast is to store more adipose tissue triacylglycerol. Hibernators, the most well adapted to fasting of all the mammals, take full advantage of this strategy. For example, woodchucks (*Marmota monax*), which fast for up to six months during the winter, accumulate fat stores prior to hibernation that can comprise more than 50% of their body mass (Snyder *et al.*, 1961). Hibernators are then able to rely almost completely on this fuel. Cherel *et al.* (1995) looked at the different energy sources used by European hedgehogs (*Erinaceus europaeus*) during a six month hibernation. During this time they lost 55% of their body mass, of which lipids accounted for 54% while 11% was protein. This means that over 90% of the total energy expended was from lipids while only about 8% was from protein.

Increased lipid stores also increase fasting resistance in non-fasting adapted mammals. Goodman *et al.* (1980) observed that the ability of a rat to use fat stores over muscle mass depended upon the extent of its fat mass. Lean rats increased their rates of nitrogen (N) excretion after 8 days of fasting, while obese rats did not increase their rate of N excretion even after 14 days of fasting. Presumably protein use (as measured by N excretion) increased in the lean rats when their fat stores had been exhausted, unlike the

obese rats which could still rely mainly on lipids. A similar study by Cherel *et al.* (1992) also measured the ability of obese and lean rats to survive an extended fast. The lean rats went from 12% of their body mass being lipid to 3% lipid in 12 days. At this point their usable lipid stores were depleted, homeostasis was lost, and their rate of weight loss and protein oxidation increased dramatically. The rate at which body mass is lost increases with a sudden increase in protein use because protein must be metabolized at twice the rate of lipid in order to maintain the same energy level. Also, because lipids can be stored dryer and lighter than proteins, the rate of weight loss when lipids are being preferentially metabolized is slower than when proteins are being preferentially metabolized. Conversely, the obese rats went from 45% lipid to 27% lipid after 81 days and at no point was there an increase in either their rate of weight loss or N excretion. Unfortunately, because obese animals do use some protein throughout the fast (albeit at a much lower rate than lean animals) very lengthy fasts can still be fatal, even if they still have an abundance of lipid reserves. Cherel *et al.* (1992) conclude that it is the low, though constant, loss of N over an extended period of time that limits an obese animal while a lean animal is limited by the loss of almost all lipid reserves forcing a rapid and pathological utilization of lean body tissue. Even humans on severe calorie-reduced diets are able to survive for a longer period of time if they have a higher body mass index (weight-to-height ratio; Van Itallie and Yang, 1984). Unfortunately, like obese rats, fasting obese humans continue to utilise protein and, even though it is at a much more reduced rate than their lean counterparts, they can suffer irreversible organ damage and death.

The third major adaptation to fasting is the ability of animals to reduce their metabolic rate, which acts to slow down weight loss and fuel utilization. Hibernators are

also probably the best examples of mammals that can use this strategy to prolong a fast. During hibernation, woodchucks drop their oxygen consumption to 4-10% of normal and their heart rate from approximately 100 beats per min to 8-10 beats per min (Lyman, 1958). Entenman *et al.* (1975) measured the *in vitro* oxidation of radioactively labeled palmitate in hibernating ground squirrel liver slices. They found that fatty acid oxidation of the hibernating hypometabolic animals was one-third that of the fatty acid oxidation of their warm euthermic counterparts. Tashima *et al.* (1970) found that the oxidation of  $^{14}\text{C}$  glucose following injection of the substrate into hibernating ground squirrels was only 16% that of active squirrels. These reductions in fuel use are mainly related to concurrent reductions in energy expenditure.

Non-hibernating animals are also able to utilise this strategy during a fast. Gray seal pups reduce their mass specific metabolic rate by 45% in response to fasting when they are weaned (Nørdoy *et al.*, 1990). Merkt and Taylor (1994) also found that when the spiny desert mouse, a fasting adapted species, is faced with a food shortage it can switch its fasting resting metabolism to almost 50% of its post-absorptive resting metabolism and as a result slow down weight loss. This is in contrast to the laboratory white mouse that can only decrease its metabolism by about 10% and loses weight very steadily (Merkt and Taylor, 1994). In fact, many mammals, including humans (Keys *et al.*, 1950), are not able to reduce their mass specific metabolic rate by much more than 20% when fasting, while others are not able to at all (*e.g.* Virginia opossum; Weber and O'Connor, 2000). Nevertheless, even a small depression of metabolic rate during a fast will help increase an individual's chances of survival.

To summarize, mammals are able to safely reduce the rate at which their body mass is lost and spare protein reserves by: 1) preferentially using fat rather than protein, 2) having large lipid stores before the fast is started and 3) having the ability to reduce energy expenditure during the fast. However, despite this knowledge of what gives an animal a greater chance of surviving a long-term fast, very little is known, besides variations in plasma metabolite concentration, about the specific changes that occur in lipid metabolism during long-term fasting, especially when energy expenditure may also be decreasing.

**Figure 1.1. Hormonal regulation of the cAMP cascade that controls triacylglycerol hydrolysis (lipolysis) in the adipocyte including the pathway through which leptin possibly mediates its effects. The thick arrows indicate a stimulatory effect.**

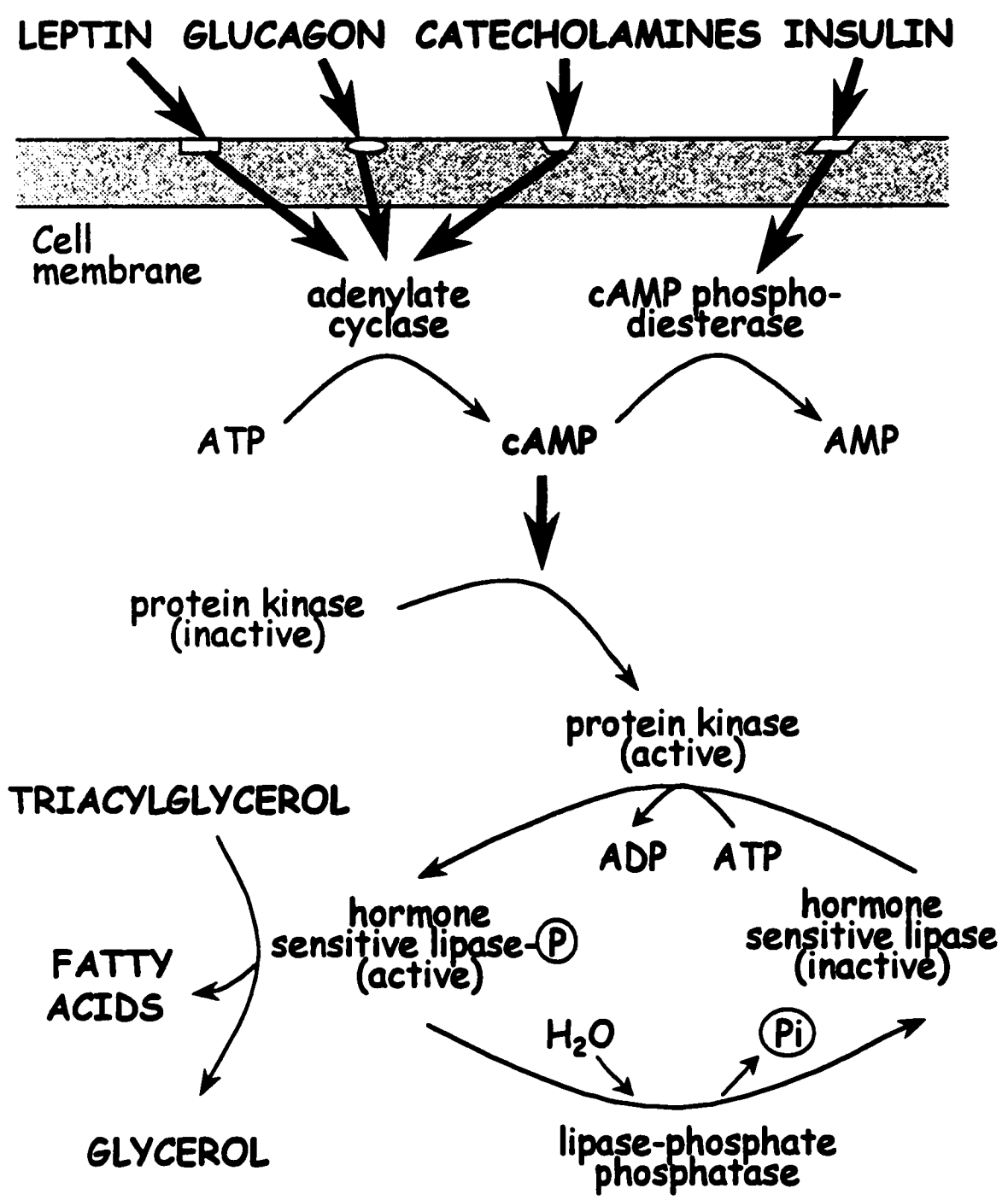


Figure 1.1

**Figure 1.2. Schematic diagram of the triacylglycerol/fatty acid (TAG/FA) substrate cycle.**

**The top cell is an adipocyte where most body triacylglycerol (TAG) is stored. TAG is composed of one glycerol and three fatty acids (FA). Some of the FA that are released from TAG by lipolysis are reesterified in the same cell. This is primary reesterification. Glycerol and the other fatty acids are mobilized into the blood where they are circulated to other tissues/cells. The rate of metabolite movement into the circulation is also called the rate of appearance (Ra) or flux. The FA can move into another cell (e.g. hepatocyte) where they are either oxidized for ATP production or reesterified (secondary) back into TAG.**

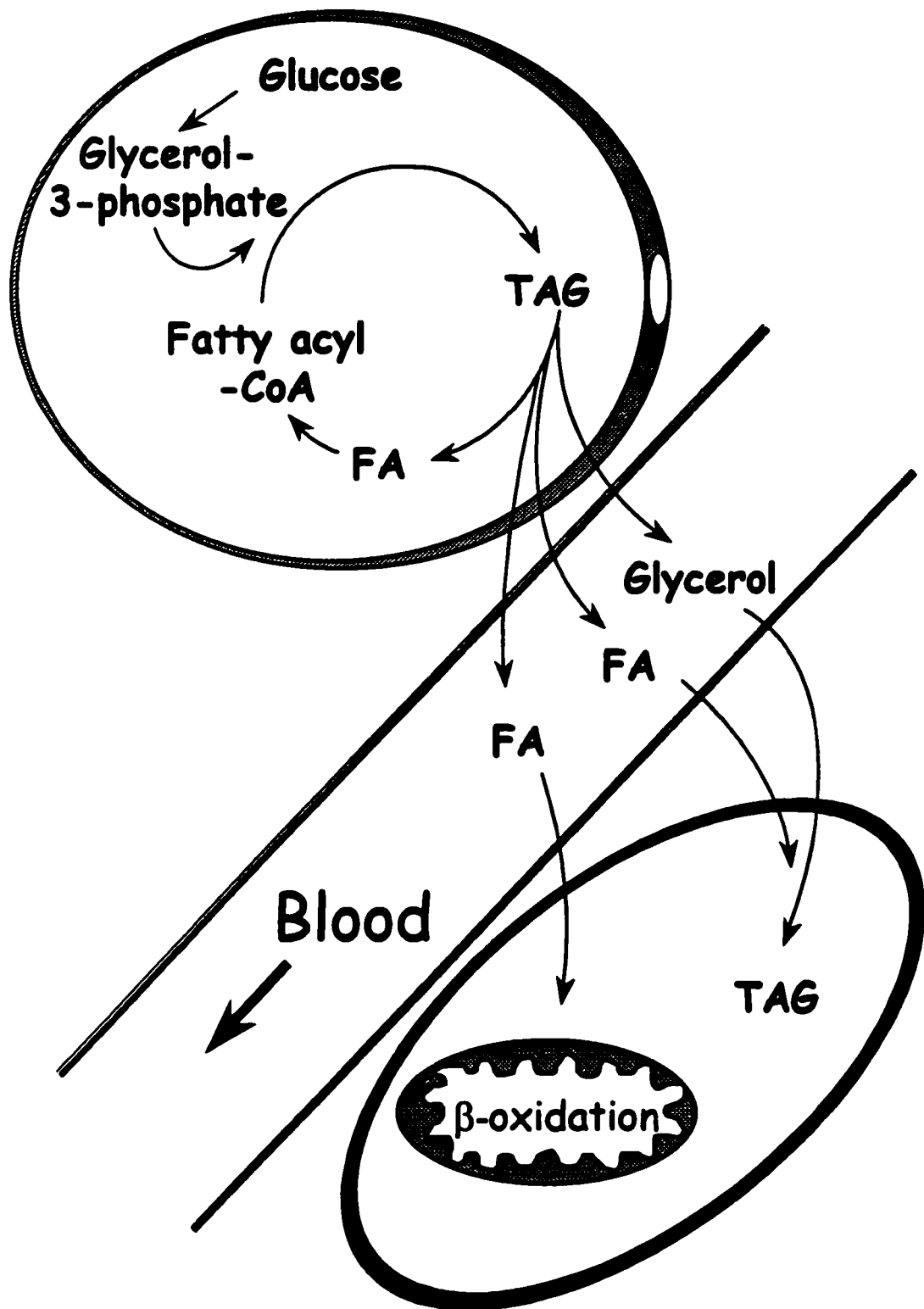


Figure 1.2

**Figure 1.3. Schematic diagram representing the pathways through which leptin has its effect on lipid metabolism, energy expenditure and caloric intake. The large circle represents any cell-type (e.g. adipocyte, myocyte). A negative sign indicates an inhibitory effect and a positive sign indicates a stimulatory effect. Although not indicated, the increase in  $\beta$ -oxidation and mitochondrial uncoupling caused by an increase in leptin levels would also act to stimulate lipolysis. The effect of leptin on the release of glucagon from pancreatic  $\alpha$ -cells is still not clear. TAG = triacylglycerol, FA = free fatty acid, CAT= catecholamines, T<sub>3</sub>= triiodothyronine.**



### *Goals of the investigation*

The general aim of this thesis was to characterize some of the effects of long-term fasting, hibernation and leptin on *in vivo* lipid metabolism (specifically lipolysis, fatty acid mobilization, and fatty acid reesterification). Two animal species (both bred in captivity for many generations) were used for this work: the woodchuck (*Marmota monax*) and the laboratory rabbit (*Oryctolagus cuniculus*). The woodchuck was used as an experimental model of a fasting adapted animal, while the rabbit was used as the experimental model of a non-fasting adapted animal. All the experimental procedures described in this thesis were approved by the Animal Care Committee of the University of Ottawa. The work is divided into the following main parts:

1. The changes in fuel metabolism and energy expenditure that occur during long-term fasting in the normothermic woodchuck and rabbit.
2. The difference in the rates of TAG/FA substrate cycling, lipolysis and fatty acid mobilization in woodchucks following arousal from a three month hibernation compared to normothermic fasting woodchucks.
3. The temporal changes in the rates of rabbit TAG/FA substrate cycling, lipolysis and fatty acid mobilization that occur with long-term fasting.
4. The effect of leptin treatment on the rates of rabbit TAG/FA substrate cycling, lipolysis and fatty acid mobilization.

## **CHAPTER 2**

### **TEMPORAL CHANGES IN FUEL METABOLISM OF A HIBERNATOR FASTING AT NORMAL TEMPERATURES**

## **Introduction**

When mammals are fasted, they quickly utilize their endogenous carbohydrate (glucose and glycogen) and begin to rely on lipid and protein. When this happens, it is advantageous to use lipids as the primary metabolic fuel and conserve rather than use body protein because protein is needed to maintain muscle function (*e.g.* heart; Garnett *et al.*, 1969; Van Itallie and Yang, 1984). As a result, lipids are the primary fuel used by fasting vertebrates regardless of their natural history. However, the amount of protein that an animal uses in proportion to fat utilization during periods of food deprivation differs widely among species. Non-fasting adapted animals, that is, animals that do not go through regular periods of fasting due to seasonal changes or other reasons, derive about 20-25% of their fasting energy from protein (Cahill, 1970; Cherel *et al.*, 1992; Goodman *et al.*, 1980; Henry *et al.*, 1988). Conversely, fasting adapted animals that regularly go through periods of fasting acquire less than 10% of their total fasting or hibernating energy from protein (Cherel *et al.*, 1995; Galster and Morrison, 1966; Lundberg *et al.*, 1976; Nørdoy *et al.*, 1990). Thus, it appears that animals considered fasting adapted are able to derive a lower percentage of their total energy expenditure from protein compared to animals that do not fast on a regular basis.

Most investigation on fuel use in hibernators, probably the best fasting adapted of all the mammals, has focused on which fuels they use during periods of hypothermia rather than at non-hibernating temperatures. However, many hibernators also fast seasonally at normothermic temperatures as they often continue their reliance on endogenous energy for weeks after they have emerged from hibernation in the spring. For example, woodchucks often arouse from hibernation weeks before the snow has receded outside of their burrow and then,

even following emergence from their burrows, they do not begin to eat for a few more weeks and continue to lose weight (Davis, 1976; Hamilton, 1934; Snyder *et al.*, 1961). Similarly, black bears do not eat for several weeks following their spring emergence even if food is available (Nelson, 1980). Nevertheless, despite the ability of these animals to fast and the hundreds of studies that have explored the physiology of hibernation, almost no information exists on the response of hibernators to fasting at normal body temperatures. Therefore, one of the primary goals of this study was to look at the fasting response and fuel use of a hibernator at normothermic temperature. Cherel *et al.* (1995) found that fasting hedgehog fuel utilization relative to energy expenditure did not differ with shallow and deep hypothermia, therefore, it seems likely that a hibernator fasting at normal temperatures in a euthermic state will also spare protein as they do during hibernation.

Although there is huge inter-specific variation in the protein catabolism of fasting animals, some protein must always be broken down to provide amino acids for gluconeogenesis. In humans, amino acids contribute to approximately one-quarter of the glucose that is formed through gluconeogenesis (Owen *et al.*, 1998; Peroni *et al.*, 1997). The remaining glucose is derived from glycerol, lactate, pyruvate and acetone (Owen *et al.*, 1998). The nitrogenous waste products of amino acid carbon skeleton oxidation are excreted in the urine as urea, ammonium, uric acid and creatinine, and as a fast progresses, many animals begin to decrease the urinary excretion of nitrogen in urea and increase its excretion in ammonia (Nørdoy *et al.*, 1990; Owen *et al.*, 1969; 1998). Therefore, I also measured the nitrogenous waste products that were produced in the urine of hibernators fasting at normothermic temperatures to see if they would similarly change with fasting time.

Another objective of this study was to see if a hibernator fasting at euthermic temperature will show metabolic depression. Even though a hibernator's basal metabolic rate changes seasonally, decreasing during the fall and winter at normal temperatures, and dropping by up to 96% during hibernation (Körtner and Heldmaier, 1995), it is still unknown if a hibernator fasting at normal temperatures will also drop energy expenditure. Among other fasting adapted and non-fasting adapted animals this is a common mechanism to reduce fuel use while fasting and prolong survival (Keys *et al.*, 1950; Ma and Foster, 1986; Merkt and Taylor, 1994). The woodchuck is a good model for this because in nature they fast in the spring at normothermic temperatures (Snyder *et al.*, 1961). Also, unlike other rodent hibernators that rely primarily on ambient air temperature to trigger entry into hibernation, woodchucks only hibernate if they are deprived of food in addition to a decrease in ambient air temperature (Goodrich, 1973). This suggests that the ability of woodchucks to decrease their metabolic rate in response to food deprivation is not entirely seasonal. The domestic rabbit was chosen for comparison as the non-fasting adapted species.

## **Methods**

### *Animals*

Male and female laboratory rabbits (*Oryctolagus cuniculus*, New Zealand white) and woodchucks (*Marmota monax*) were used in this study. The rabbits were provided by Charles River (St. Hyacinthe, QC, Canada) and the woodchucks came from a large captive colony maintained at Cornell University (Ithaca, NY, USA). The rabbits were fed rabbit chow (approximately 52% carbohydrate, 3% fat, 16% protein, 14% crude fiber, 15% moisture) and the woodchucks were fed woodchuck pellets (approximately 54%

carbohydrate, 2% fat, 15% protein, 18% crude fiber, 11% moisture). The fasting experiments for the woodchucks were initiated in early spring (February, n=1 and March, n=3) and summer (July, n=2 and August, n=2). The woodchucks had not hibernated the previous winter. The rabbits (n=9) were fasted at different times between March and October.

### *Experimental design*

A single animal was contained in a closed Plexiglas respirometer (54cm x 38cm x 67 cm) supplied with room air at 3-8 l min<sup>-1</sup> depending upon its metabolic rate. A small enclosed fan in the respirometer lid circulated the air inside the chamber. For the first 3 days of the experiment the animal was given free access to food and water and then only water was provided. For the rabbits this continued until either 7 days had passed or the rabbit had lost 15% of its initial body mass. For the woodchucks, the fast was stopped when either 14 days had passed or the animal had lost 30% of its body mass. The Animal Care Committee of the University of Ottawa imposed these values of maximal weight loss and fasting time. Continuous 24 h measurements of whole animal oxygen consumption and carbon dioxide production were recorded using an indirect calorimetry system (Columbus Instruments; Columbus, Ohio, USA) connected to the respirometer (described by Fournier and Weber, 1994). Every 24 h the respirometer was cleaned, and the oxygen and carbon dioxide sensors were calibrated using gases of known concentrations. Water and food consumption, feces and urinary excretion, weight and body temperature were recorded. Their temperature was measured with a rectal digital thermometer. The urine was collected as it passed through the metal floor of the respirometer and funneled into an

attached polyethylene bottle kept on ice. To help prevent bacterial growth, a crystal of thymol was added to the bottle before urine collection was started for the day.

### *Analyses and calculations*

Total urinary nitrogen excretion was measured by the Kjeldahl method using Tecator Analysers (1007 Digester and Kjelttec System 1002 Distilling Unit). This method converts all the nitrogen in a sample into ammonium sulfate by digestion with a sulfuric acid mixture in the presence of sodium thiosulfate, potassium sulfate and copper sulfate. Excess alkali is then used to distill the ammonium sulfate and the free ammonia is collected in a known excess quantity of acid. This acid-ammonia mixture is then titrated with alkali to determine the ammonia concentration.

Urea concentration was determined using the method described by Bergmeyer (1985). Briefly, urease (Sigma, St. Louis, MO, USA) was used to convert the urea to ammonia and then glutamate dehydrogenase (Sigma, St. Louis, MO, USA) was used as an indicator enzyme to cause a change in NADH concentration depending upon how much glutamate was formed from the ammonia. This change was detected as a change in light absorbance using a Beckman DU 640 spectrophotometer at 340 nm. Uric acid concentration was measured using the enzyme uricase. In this assay, uric acid has a characteristic absorption at 292 nm, however, as it is converted to allantoin by uricase this absorption changes and can be measured using the spectrophotometer (Bergmeyer, 1985). Creatinine concentration of the urine was colorimetrically determined at 500 nm using a Sigma diagnostics kit (Sigma, St. Louis, MO, USA). Total ammonia ( $\text{NH}_3$  plus  $\text{NH}_4^+$ ) concentration was determined using a method described by Verdouw *et al.* (1978). Using

sodium nitroprusside as the catalyst, a blue colour is produced when ammonia reacts with salicylate and hypochlorite. Changes in this colour can be measured at 650 nm with a spectrophotometer.

For the fed and one day fasted values, rates of lipid, protein and carbohydrate oxidation were calculated according to Frayn (1983):

$$\text{Glucose oxidation (g min}^{-1}\text{)} = 4.55 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 3.21 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 2.87 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

$$\text{Lipid oxidation (g min}^{-1}\text{)} = 1.67 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 1.67 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 1.92 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

The second equation assumes that all lipid oxidation is of palmitoyl-stearoyl-oleoyl-glycerol, the average fatty acid found in human adipose tissue. Assuming this is correct, oxidation rates were then converted to their molar equivalent by using the molecular weight of this triacylglycerol (861 g mol<sup>-1</sup>) and glucose (180 g mol<sup>-1</sup>). Finally, total urinary N excretion was used to estimate daily protein catabolism by using a value of 6.25 g of protein oxidized per g of excreted nitrogen (Frayn, 1983).

Following one day of fasting, the contribution of lipid oxidation to the total oxygen consumption of the animal was assumed to be all the non-protein oxidation. This is because with long-term fasting there is an increase in gluconeogenesis and ketone body formation and utilization (Baba *et al.*, 1995; Krilowicz, 1985; see discussion). These processes change the formation and utilization of CO<sub>2</sub> and O<sub>2</sub> and violate some of the assumptions of indirect calorimetry (Frayn, 1983; Owen *et al.*, 1998).

## *Statistics*

Statistical differences for changes during fasting were tested for by using a repeated measures analysis of variance (ANOVA) with time, individual and season or species as the main factors. When significant changes with time were detected, the Dunnett post-hoc test was used to determine which means were different from the fed value. When significant changes with fasting due to either season or species were detected by the repeated measures ANOVA, a one-way ANOVA was used to determine at what time the differences were occurring. Student t-tests were used to identify statistical differences not associated with time. All data expressed as percentages were transformed to the arcsine of their square root before testing for differences (Zar, 1984). Statistical significance is indicated when  $p < 0.05$ . Results are presented as means  $\pm$  SEM.

## **Results**

### *Seasonal differences in woodchucks*

The woodchucks did not seasonally differ in their food consumption, water consumption or body temperature; however, the summer woodchucks did weigh more ( $p < 0.05$ ; Table 2.1). When they were fasted they did not seasonally differ in their rate of weight loss (Fig 2.1a, b), although they both lowered their rate of weight loss as the fast progressed ( $p < 0.05$ ; Fig. 2.1b). On the first day of the fast their average rate of weight loss was  $17.26 \pm 1.68 \text{ g kg}^{-1} \text{ day}^{-1}$ , however, it decreased during the fast until it reached a low of only  $5.23 \pm 0.61$  on the last day of the fast (Fig 2.1b). Therefore, after two weeks of fasting the woodchucks had only lost 13% of their initial body mass (Fig 2.1a).

There was a difference in woodchuck energy expenditure with season. Oxygen consumption of the summer woodchucks was 35% higher than that of the spring woodchucks ( $p < 0.05$ ; Table 2.1). However, when the summer woodchucks were fasted they significantly reduced their metabolic rate by 25% so by the end of the fast this difference no longer existed (Fig. 2.2a, b). The spring woodchucks did not lower their metabolic rate with fasting (Fig. 2.2a, b). The change in metabolic rate observed with the summer-fasted woodchucks with fasting was also reflected in a decrease in their body temperature ( $p < 0.05$ ; Fig. 2.3), and a correlation existed between these two variables (Pearson correlation coefficient=0.595;  $p < 0.05$ ).

Because the oxygen consumption of the two groups of woodchucks differed, it is necessary to look at the relative contribution of the different metabolic fuels to total energy expenditure. The contribution of lipid (fatty acids) and carbohydrate (glucose) oxidation to woodchuck oxygen consumption before the fast was initiated did not differ with season (Fig. 2.4a). However, the summer woodchucks had a significantly higher rate of protein oxidation than the spring woodchucks. As expected, when the fast was started lipid oxidation quickly became the primary source of energy for both groups of woodchucks, while the importance of carbohydrate oxidation dropped (Fig. 2.4b). The seasonal difference in protein oxidation disappeared when the fast was started because protein oxidation by the summer woodchucks dropped. From day 2 to day 7 of the fast lipid was the primary source of energy with only a small contribution from protein for both groups of woodchucks (Fig. 2.4c).

### *Differences between woodchucks and rabbits*

Rabbit metabolic rate did not vary with season, however, there were large differences in oxygen consumption between species. Both the summer and spring woodchuck metabolic rate were lower than that of the rabbits ( $p < 0.05$ ;  $200.21 \pm 17.44$  and  $270.74 \pm 20.85$  vs.  $383.07 \pm 11.35 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ , respectively). Because the summer woodchuck metabolic rate was closer to the rabbits than the spring woodchucks, all comparisons between woodchucks and rabbits were done using the summer woodchucks. Rabbits did not consume more food than woodchucks, although their water consumption and body temperature were both higher ( $p < 0.05$ ; Table 2.1).

Fasting rabbits lost weight at a greater rate than the woodchucks ( $p < 0.05$ ; Fig 2.1a). On the first day of the fast the average rate of weight loss for the rabbits was  $34.39 \pm 4.99 \text{ g kg}^{-1} \text{ day}^{-1}$  compared to  $17.26 \pm 1.68 \text{ g kg}^{-1} \text{ day}^{-1}$  for the woodchucks. However, the rate of weight loss decreased significantly with time so that the average rate of rabbit weight loss was only  $16.32 \pm 5.48$  when the fast was terminated ( $p < 0.05$ ; Fig 2.1b). Regardless of this rate decrease, after only seven days the rabbits had lost 15% of their body mass compared to only about 8% by the woodchucks (Fig 2.1a). This difference in weight loss was coupled with a large species difference in adipose tissue size. Carcass analysis of a sub-sample of rabbits and woodchucks showed that the woodchucks had two to four times more fat relative to body mass than the rabbits ( $p < 0.05$ ; Table 2.2).

As with the summer-fasted woodchucks, rabbit oxygen consumption also decreased with fasting ( $p < 0.05$ ; Fig 2.2a,b). By the end of the seven day fast their oxygen consumption had dropped 32%, from  $383.07 \pm 11.35 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$  to  $259.16 \pm 24.74 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ . This change in metabolic rate with fasting was correlated with a

small reduction in body temperature (Pearson correlation coefficient=0.786;  $p<0.05$ ).

Mean body temperature dropped almost 1 degree from  $39.30 \pm 0.31$  °C when fed to  $38.36 \pm 0.14$  °C after seven days of fasting ( $p<0.05$ ; Fig. 2.3).

The contribution of lipid and carbohydrate oxidation to total oxygen consumption did not differ between fed woodchucks and rabbits, however, protein oxidation was higher in the rabbits ( $p<0.05$ ; Fig. 2.4a). When the fast was initiated, carbohydrate and lipid oxidation still did not differ between the rabbits and woodchucks, although protein oxidation remained greater during the entire fast ( $p<0.05$ ; Fig. 2.4a, b). This was despite the fact that the rabbits reduced their urinary nitrogen excretion and protein oxidation with fasting ( $p<0.05$ ; Fig. 2.4 and 2.5e).

#### *Urinary nitrogen excretion*

Species differences in urine volume also existed ( $p<0.05$ ; Fig. 2.6b). This was primarily due to a difference in water consumption before and during the fast ( $p<0.05$ ; Fig. 2.6a). Total urinary nitrogen excretion of feeding rabbits, independent of urine production, was  $27.37 \pm 2.69$   $\mu\text{mol N kg}^{-1} \text{ min}^{-1}$  compared to  $12.26 \pm 2.13$   $\mu\text{mol N kg}^{-1} \text{ min}^{-1}$  for the summer woodchucks and  $3.61 \pm 1.54$   $\mu\text{mol N kg}^{-1} \text{ min}^{-1}$  for the spring woodchucks ( $p<0.05$ ; Fig 2.5e). Rabbit nitrogen excretion decreased about 67% during seven days of fasting to  $8.93 \pm 1.15$   $\mu\text{mol kg}^{-1} \text{ min}^{-1}$  ( $p<0.05$ ; Fig 2.5e).

Seasonal differences in the nitrogenous composition of woodchuck urine relative to total nitrogen concentration were not significant so the woodchuck data in Figure 2.7 is an average for all the woodchucks. However, differences did exist between the rabbits and woodchucks. The contribution of uric acid to the total N content of rabbit urine was

higher than its contribution to the total N content of woodchuck urine both before and during the fast ( $p < 0.05$ ; Fig. 2.7d). Conversely, the contribution of ammonia to the total N content of woodchuck urine was higher than its contribution to rabbit urine before and during the fast ( $p < 0.05$ ; Fig. 2.7b). The contribution of urea and creatinine to total N excretion were not different between species (Fig. 2.7a,c). As total nitrogen excretion decreased with fasting (Fig 2.7e), so did the percent contribution of urea, by 22% and 10% after seven days of fasting for the woodchucks and rabbits respectively ( $p < 0.05$ ; 2.7a). The contribution of ammonia to total nitrogen excretion of the woodchucks doubled during the fasting period ( $p < 0.05$ ; Fig 2.7b). A similar increase did not occur with the rabbits, the contribution of ammonia to their total urinary nitrogen content remained around 1%.

Table 2.1. Body mass, oxygen, food and water consumption, fecal (wet weight) and urinary excretion and body temperature of fed rabbits and woodchucks after 3 days in the respirometer. Values are means  $\pm$  SEM and \* indicates a significant difference between the marked value and the value to its left.

	Woodchuck		
	Spring	Summer	Rabbit
N	4	4	9
Body mass (kg)	3.13 $\pm$ 0.31	4.195 $\pm$ 0.28 *	3.052 $\pm$ 0.08 *
Mean 24 h oxygen consumption ( $\mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ )	200.2 $\pm$ 17.44	270.7 $\pm$ 20.85 *	383.1 $\pm$ 11.35 *
Food consumption ( $\text{g kg}^{-1} \text{ day}^{-1}$ )	18.77 $\pm$ 4.43	28.23 $\pm$ 9.29	28.30 $\pm$ 3.36
Water consumption ( $\text{ml kg}^{-1} \text{ day}^{-1}$ )	8.93 $\pm$ 2.41	4.99 $\pm$ 2.78	72.01 $\pm$ 12.12 *
Fecal excretion ( $\text{g kg}^{-1} \text{ day}^{-1}$ )	6.07 $\pm$ 4.02	8.48 $\pm$ 1.88	11.13 $\pm$ 2.03
Urine volume ( $\text{ml kg}^{-1} \text{ day}^{-1}$ )	13.36 $\pm$ 5.81	18.34 $\pm$ 2.93	45.34 $\pm$ 6.56 *
Body temperature ( $^{\circ}\text{C}$ )	36.75 $\pm$ 0.32	36.45 $\pm$ 0.61	39.12 $\pm$ 0.22 *

Table 2.2. Body composition of a sub-sample of fed rabbits and woodchucks as determined by carcass dissection. Values are means  $\pm$  SEM and \* indicates a significant difference between the marked value and the value to the left. LBM = lean body mass.

	Woodchuck			Rabbit
	Spring	Summer		
N	2	2		3
Body mass (kg)	2.828 $\pm$ 0.372	3.955 $\pm$ 0.200		3.633 $\pm$ 0.09
Skeletal muscle (% LBM)	52.41 $\pm$ 1.19	54.32 $\pm$ 1.59		48.23 $\pm$ 0.70 *
Adipose tissue (% body mass)	40.31 $\pm$ 0.53	56.10 $\pm$ 0.48 *		17.85 $\pm$ 1.80 *
Heart (% LBM)	1.10 $\pm$ 0.09	1.17 $\pm$ 0.01		0.42 $\pm$ 0.02 *
Liver (% LBM)	6.08 $\pm$ 0.11	6.16 $\pm$ 0.44		3.10 $\pm$ 0.37 *

Figure 2.1. Woodchuck and rabbit a) body mass (expressed as a percentage of their initial body mass) and b) rate of mass loss ( $\text{g kg}^{-1} \text{min}^{-1}$ ) before (time=0) and during a fast. Day zero is the fed value before the fast was initiated. Circles indicate summer woodchucks (n=4), squares indicate spring woodchucks (n=4) and triangles indicate rabbits (n=9). Values are means  $\pm$  SEM.

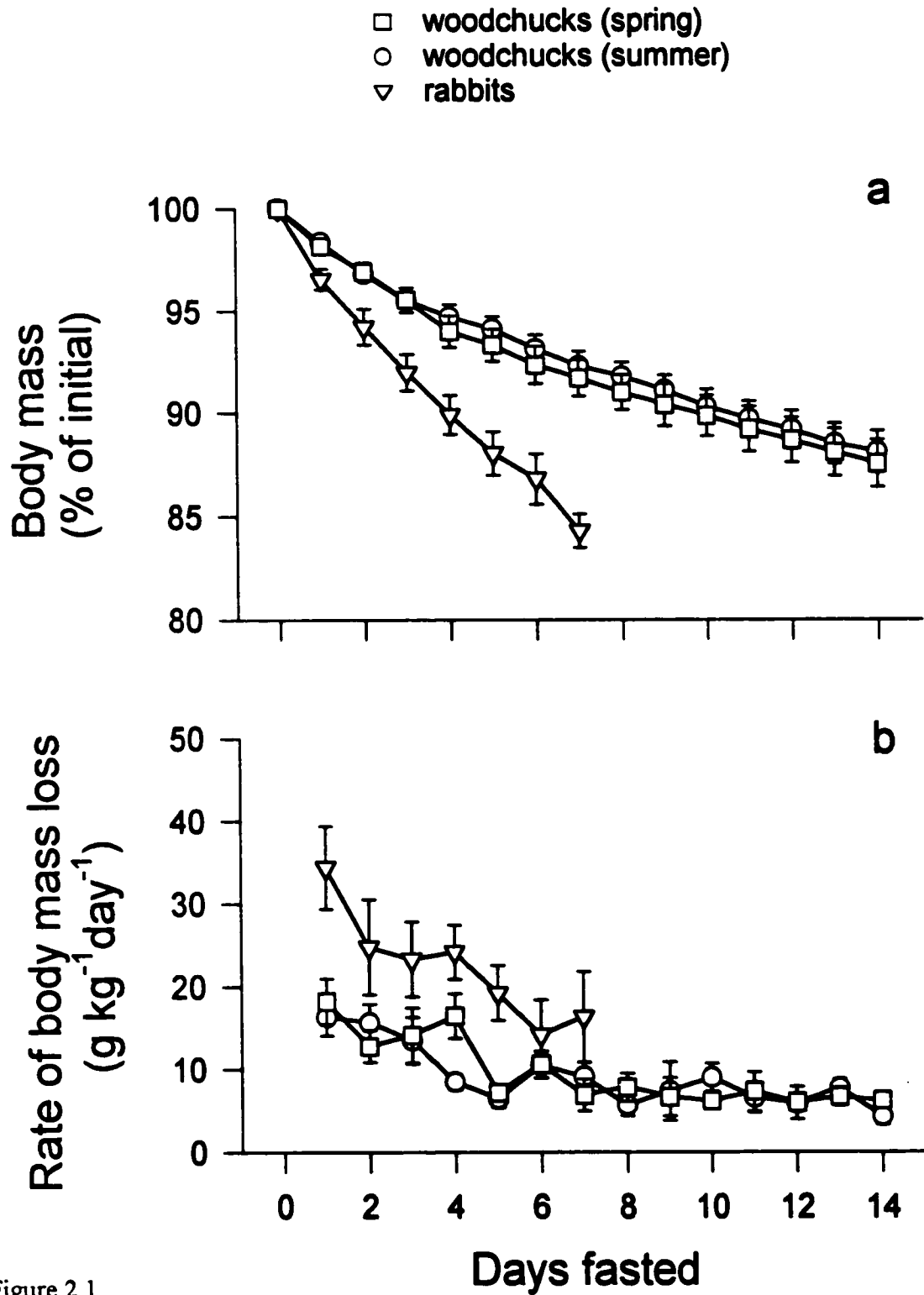


Figure 2.1

**Figure 2.2. Twenty-four hour total oxygen consumption of fasting woodchucks and rabbits expressed a) in absolute values ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) and b) relative to the initial fed value. Day zero is the fed value. Circles indicate summer woodchucks (n=4), squares indicate spring woodchucks (n=4) and triangles indicate rabbits (n=9). Values are means  $\pm$  SEM.**

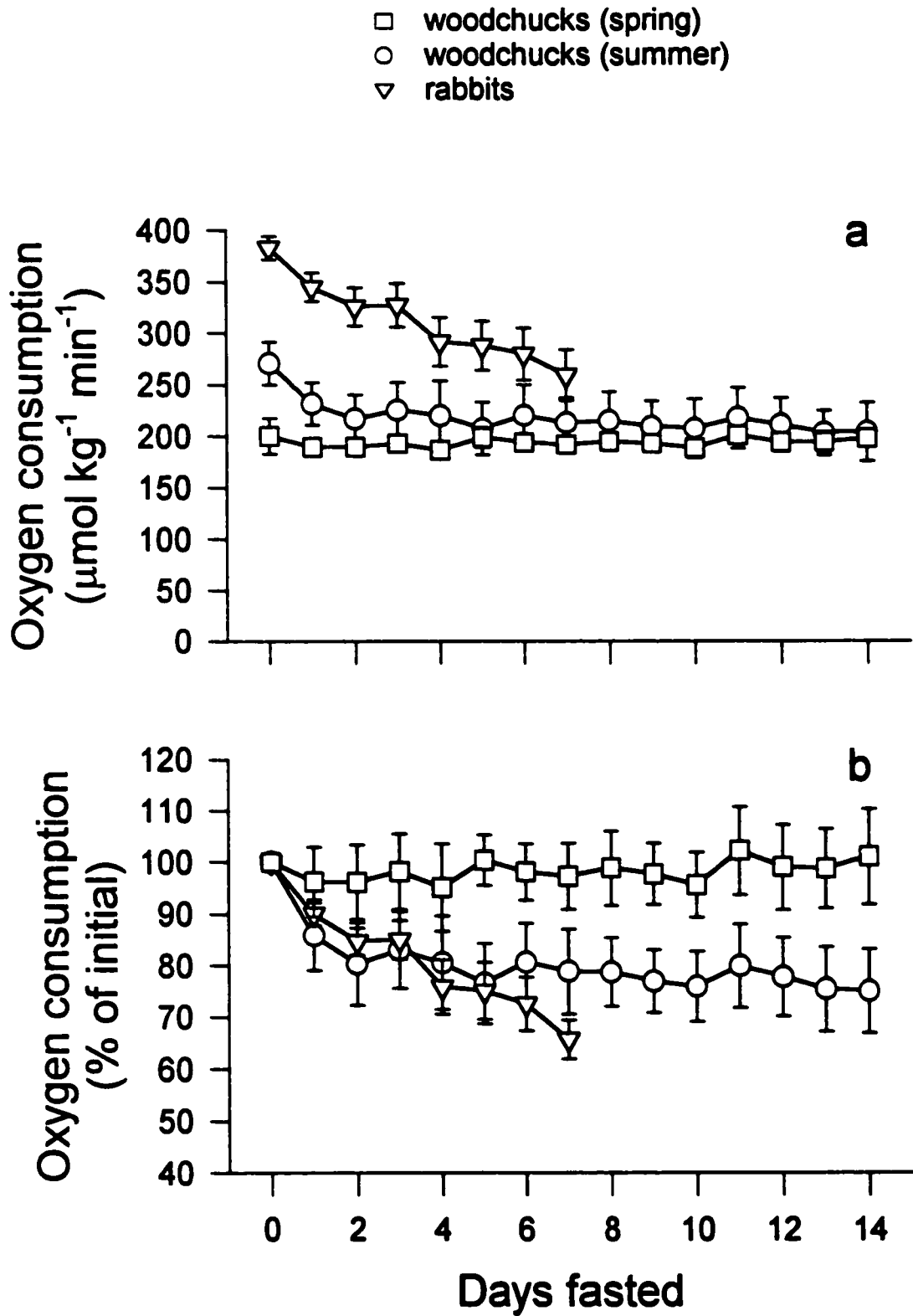


Figure 2.2

**Figure 2.3. Woodchuck and rabbit body temperature before (time=0) and during a fast.**

**Circles indicate summer woodchucks (n=4), squares indicate spring woodchucks**

**(n=4) and triangles indicate rabbits (n=9). Values are means  $\pm$  SEM and \* indicates a significant difference from the fed value for that group.**

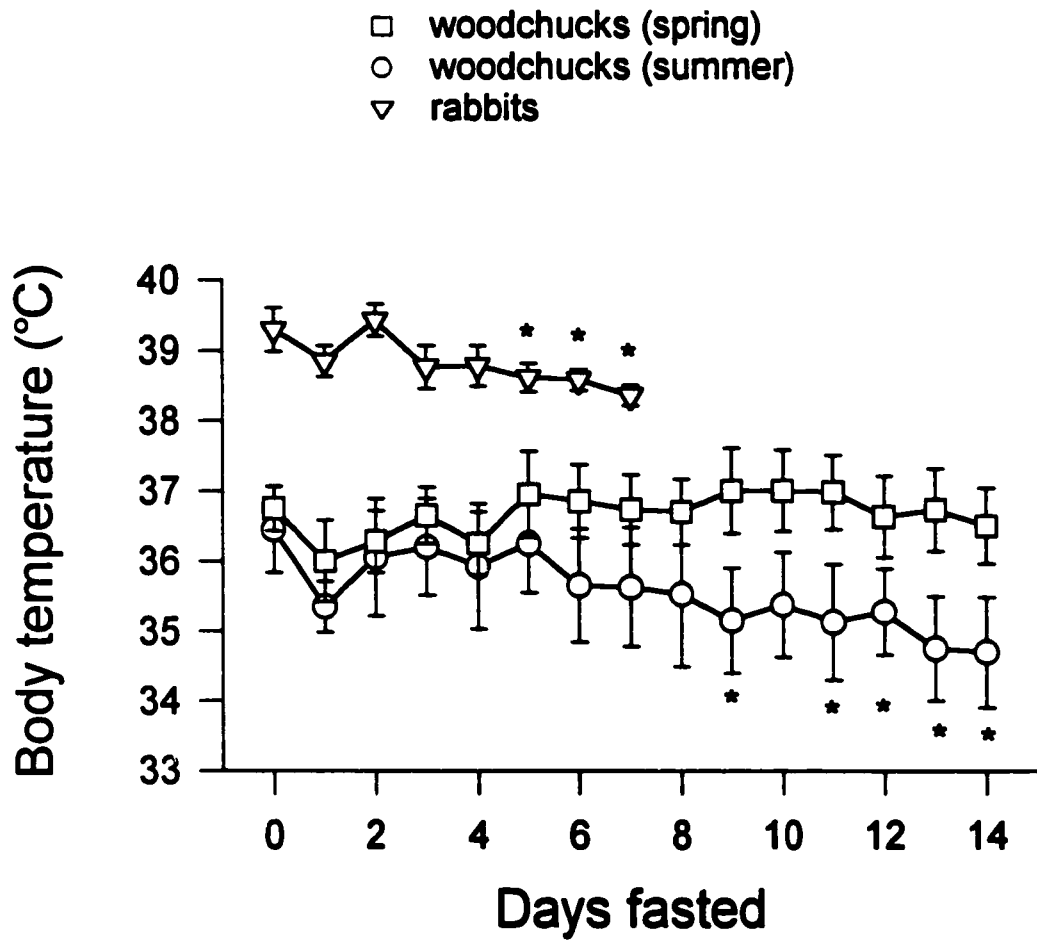


Figure 2.3

**Figure 2.4. Contribution of the different metabolic fuels to the total aerobic energy expenditure of woodchucks (n=4) and rabbits (n=9) before and during a seven day fast. Values of the same fuel type that are marked with the same symbol are significantly different from each other. Values are means  $\pm$  SEM.**

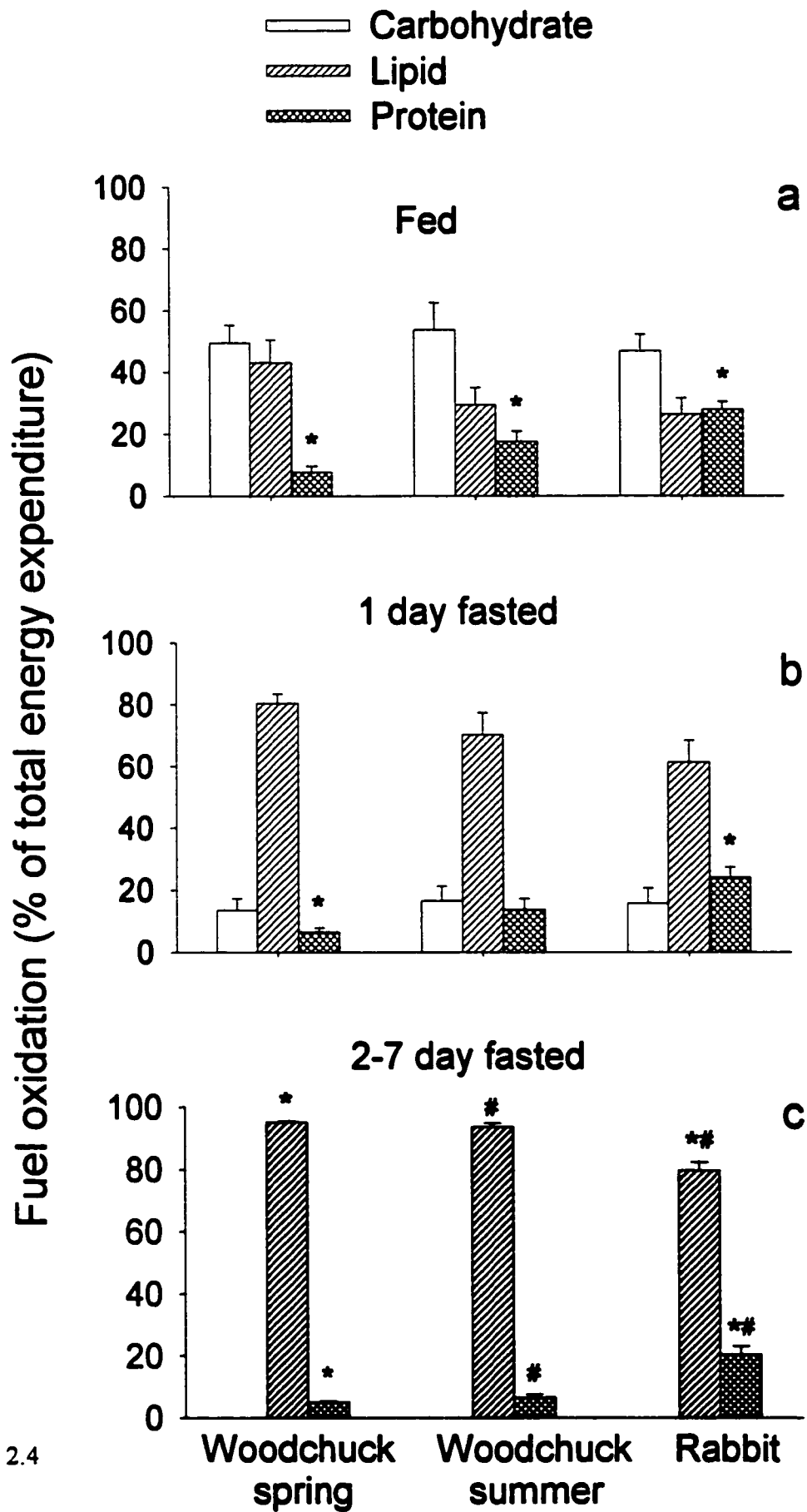


Figure 2.4

**Figure 2.5. Woodchuck and rabbit a) water consumption ( $\text{ml kg}^{-1} \text{ day}^{-1}$ ) and b) urine production ( $\text{ml kg}^{-1} \text{ day}^{-1}$ ) before (time=0) and during a fast. Circles indicate summer woodchucks (n=4), squares indicate spring woodchucks (n=4) and triangles indicate rabbits (n=9). Values are means  $\pm$  SEM.**

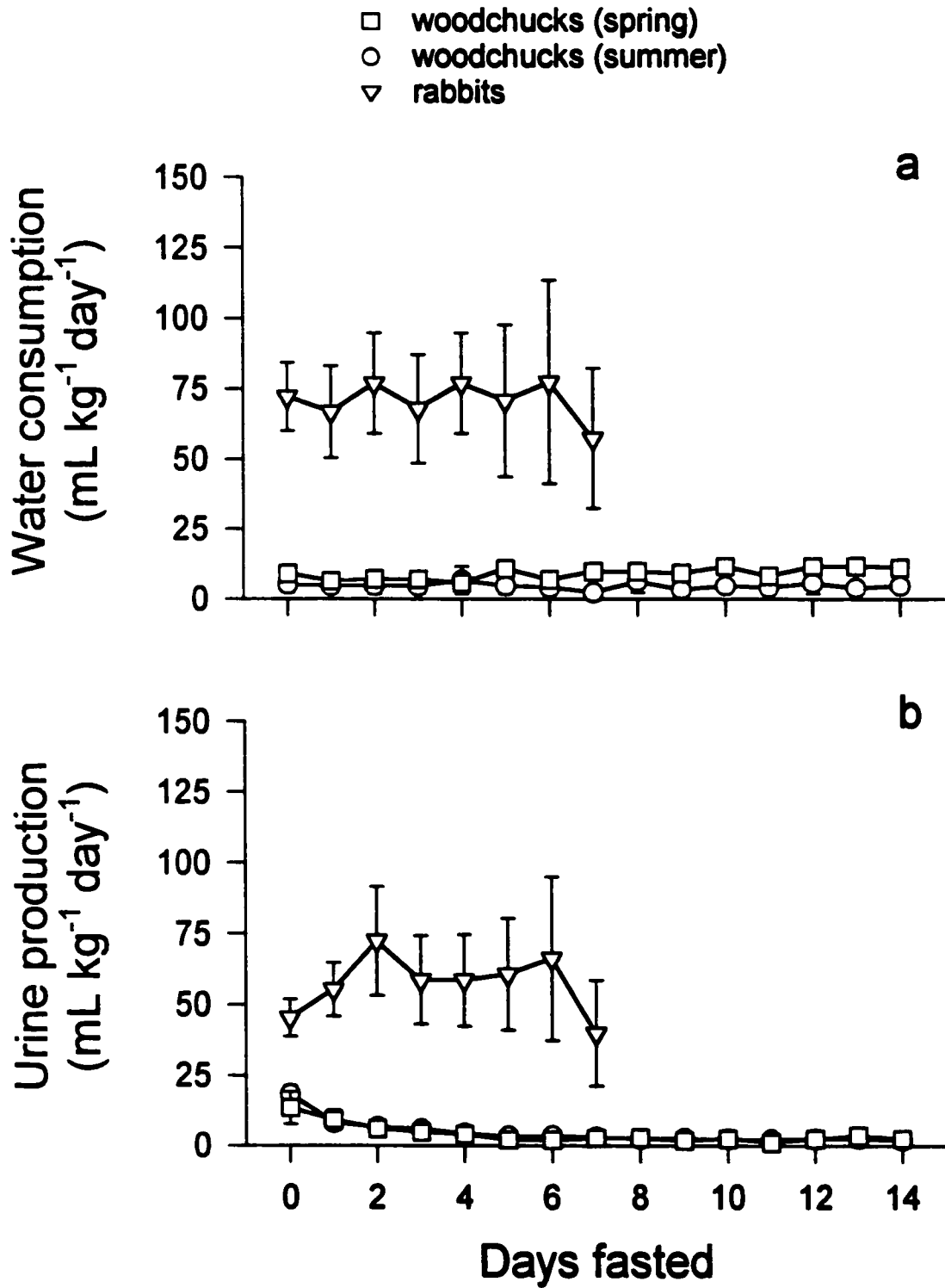


Figure 2.5

Figure 2.6. Woodchuck and rabbit urinary excretion rate of a) urea b) total ammonia c) creatinine and d) uric acid expressed as a percentage nitrogen contribution to e) total nitrogen ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) before (time=0) and during a fast. Woodchuck means include both spring and summer woodchucks. Open circles indicate rabbits (n=9) while solid circles indicate woodchucks (n=8). Values are means  $\pm$  SEM and \* indicates a significant difference between that value and the fed value.

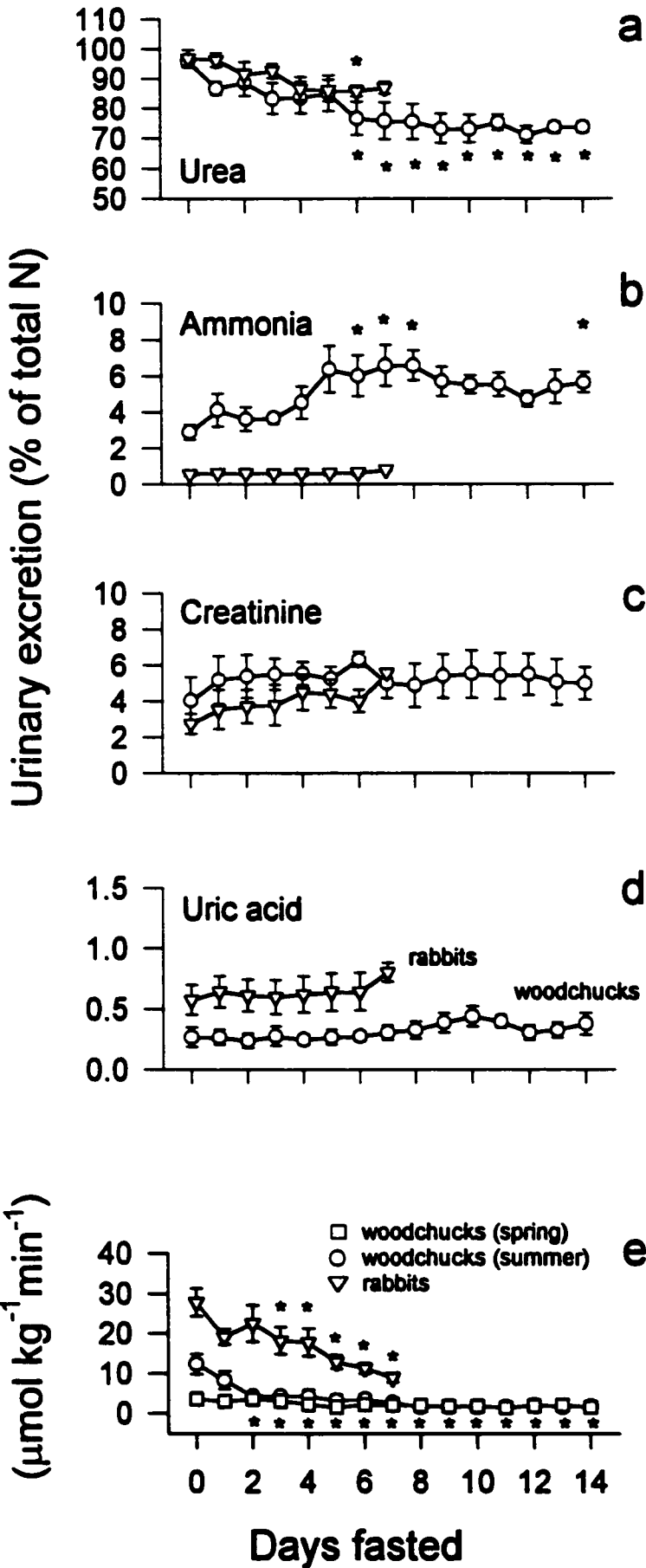


Figure 2.6

## Discussion

The oxygen consumption of summer woodchucks measured in this study is similar to the oxygen consumption of woodchucks measured by Rawson *et al.* (1998) who also looked at seasonal differences in woodchuck oxygen consumption. They measured a mean oxygen consumption rate of  $196.43 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$  on woodchucks in late September and early October, and  $325.89 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$  at the same time of the year on woodchucks acclimatized to a reverse photoperiod. With a reverse photoperiod the metabolism of these woodchucks would be approximately similar to that of woodchucks in April. Obviously, those woodchucks were already approaching their yearly peak in metabolism as their oxygen consumption was already 67% higher than the fall (boreal) woodchucks in the same study. My July-August woodchucks fell between the two with an oxygen consumption of  $270.7 \mu\text{mol O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ . Woodchuck metabolic rate has long been known to start a seasonal decline at this time of year prior to their entry into hibernation (Bailey, 1965; Körtner and Heldmaier, 1995), so my woodchucks were probably starting to lower their metabolic rate. No measurements of oxygen consumption have been made on non-hibernating woodchucks in early spring before the seasonal increase in metabolic rate commences, although Bailey *et al.* (1965) did measure the monthly changes in  $\text{CO}_2$  production of 24 hr fasted woodchucks. In that study, average woodchuck  $\text{CO}_2$  production in February/March was 20% lower than in July/August. In this study, the difference in metabolic rate for those same months is 26%. Therefore, it appears that I was in fact able to measure the metabolic activity of woodchucks in the spring just prior to their seasonal increase in oxygen consumption. Low energy expenditure after their final arousal from hibernation is probably critical for helping them

cope with the additional fasting period that they face after already not eating for five months in a hypometabolic state. The spring woodchucks in this study also had a very low rate of protein oxidation. It was about one-half the rate measured in the summer, and dropped even more when they were fasted. This is probably an additional mechanism at this time of the year to help the woodchucks protect their muscle mass.

In the summer, wild woodchucks are usually faced with an unlimited source of food, although this study shows that if they are faced with a fast at the end of the summer they can drop their metabolic rate, which is already significantly lower than other similarly sized mammals, as a mechanism to save energy. In addition to reducing their metabolic rate, woodchucks fasting in the summer are able to dramatically lower the rate at which they utilize protein, essentially conserving their muscle mass while fasting.

Other fasting adapted mammals lower their metabolic rate and spare their protein reserves when they are fasting. Many seal species fast as adults during the breeding season and while nursing, and as pups between when they are weaned and start eating solid food. During this time they can reduce their mass-specific metabolic rate by up to 45%, and obtain less than 6% of their expended energy from protein oxidation (compared to ~10% before a fast) (Nørdoy *et al.*, 1993; 1990; Worthy and Lavigne, 1987). Desert and temperate (non-hibernating) animals are also faced with periods of food restriction in their habitat due to either drought or snow cover, respectively. Two desert species, the spiny desert mouse (*Acomys russatus*) and the Bedouin goat (*Capra hircus*), both survive by having the ability to lower their metabolic rate to 50% of their post-absorptive resting metabolism (Choshniak *et al.*, 1995; Merkt and Taylor, 1994). Unfortunately, little information is available on how non-hibernating temperate animals metabolically adjust to decreased

seasonal food intake and quality with over-wintering, although it has been shown that the metabolic rate of captive white-tailed deer (*Odocoileus virginianus*) drops by 22% during the winter even when plenty of food is provided (Silver *et al.*, 1969; Worden and Pekins, 1995).

Bears are an example of the ultimate seasonal faster. In the winter, black bears (*Ursus americanus*) and grizzly bears (*U. arctos*) enter a 'pseudo-hibernation', described as such because their body temperature only drops a few degrees and they remain alert (Watts *et al.*, 1981). Then for up to six months of fasting, they lower their metabolic rate by about 30% and, amazingly, their rate of protein oxidation not only falls to zero but they can increase lean body mass (Barboza *et al.*, 1997; Nelson *et al.*, 1973; Nelson, 1973; 1980). Polar bears (*U. maritimus*) also have the ability to lower the contribution of protein to their total energy expenditure to as low as 1% (Atkinson *et al.*, 1996; Nelson, 1987). This occurs during the summer when the pack ice has receded and they do not have access to their prey (seals), and, unlike other bears, they remain physically active during the fast.

The large decrease in rabbit energy expenditure and protein oxidation with food deprivation was not surprising considering that many non-fasting adapted mammals also have the ability to reduce their metabolic rate when fasting, although to a much lesser degree than fasting adapted species. For example, fasting humans and laboratory mice and rats can decrease their metabolic rate by 10-26% depending upon the species (Keys *et al.*, 1950; Ma and Foster, 1986; Merkt and Taylor, 1994), and lower their rate of protein oxidation (Cahill, 1970; Goodman *et al.*, 1980). The rabbits, however, had a very high initial rate of protein oxidation (accounting for about 28% of total oxygen consumption). Similar high rates of nitrogen excretion have previously been measured in fasting rabbits

(Hannaford *et al.*, 1982). However, mammalian rates of protein oxidation usually range from 10 to 25%, even for animals that consume a diet high in protein (Forslund *et al.*, 1999; Henry *et al.*, 1988; Weber and O'Connor, 2000). One reason protein oxidation may be so high for rabbits is because they are coprophagic. Their feces consist of two types, a soft feces, which they consume directly from their anus, and a hard feces, which they let pass. The soft feces has a protein content that is 21% higher than the protein content of the hard feces and if they are prevented from eating the soft feces rabbits digest 33% less protein and retain 42% less nitrogen compared to when they can consume their feces (Thacker and Brandt, 1954).

There was a correlation between body temperature and a decreasing metabolic rate for both the rabbits (non-fasting adapted) and the woodchucks (fasting adapted). This is not unusual, even for non-hibernators, as decreases in body temperature are commonly associated with a reduced metabolic rate (*e.g.* rat; Ma and Foster, 1986). It is often difficult to determine what is the cause and what is the effect with body temperature and metabolic rate. However, the hormonal response to a fast includes a drop in plasma triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) concentration, the binding capacity of their receptors and (Ahima *et al.*, 1996; Rothwell *et al.*, 1982; Schussler and Orlando, 1978). Therefore, it is likely that the reduction in body temperature observed with fasting was just a consequence of decreased cellular thermogenesis.

What was particularly interesting in this study was that the animals that were the most hypometabolic (spring woodchucks) did not depress their energy expenditure at all when fasted, while the animals with the highest metabolic rate (rabbits) had the largest reduction in oxygen consumption with fasting. This raises the question of whether or not

metabolic flexibility at normal temperatures is somewhat dependent upon existing metabolic rates? Another group of animals with low resting metabolic rates are the marsupials. Interestingly, Virginia opossum (*Didelphis virginiana*) also do not lower their metabolic rate when they are fasted (Weber and O'Connor, 2000). Whether or not that is due to a minimum metabolic rate or something else (e.g. increased activity searching for food) is not known.

The size of an animal's adipose tissue mass before fasting appears to be important to how long an individual can fast (see Chapter 1). When lean rats are fasted for an extended amount of time they eventually increase their nitrogen excretion (an indirect measure of protein oxidation), indicating a severe loss of lipid reserves that has forced a rapid and pathological utilization of lean body tissue (Cherel *et al.*, 1992; Goodman *et al.*, 1980). Such an increase in protein utilization does not occur in obese rats. Fuel oxidation was not measured in these studies, but for most of the fast (up until the late-stage increase) there was no difference in nitrogen excretion between the lean and obese rats. This suggests that the size of an animal's lipid stores may only affect fuel utilisation once the fat has been pretty much depleted. One difference between the fasting adapted species and the non-fasting adapted species in this study was the capacity for the woodchucks to derive more of their total fasting energy from lipids and less from protein compared to the rabbits. Carcass analysis of a sub-sample of summer and spring woodchucks showed that there was a large difference in the size of their adipose tissue depots (Table 2.2). The woodchucks had two to four times more fat than the rabbits, however, the studies on lean and obese rats suggest that this probably did not contribute to observed species differences in fuel utilisation. Nevertheless, had I let the rabbits

fast for as long as the woodchucks, I too may have observed a similar late-stage increase in their protein oxidation.

The primary glucose source for post-absorptive animals is glycogen, however, this becomes depleted following only one day of fasting (Goodman *et al.*, 1990; Nilsson and Hultman, 1973). When this occurs, glucose requirements drop as most cells switch to fatty acid oxidation as their energy source. In this study, the indirect calorimetry method was used to measure the temporal changes in fuel oxidation that occur in fasting woodchucks and rabbits. Not surprisingly, these measurements showed a switch from carbohydrate to lipid oxidation at the very beginning of the fast. However, because some cells are unable to use fatty acids they must rely on glucose formed from gluconeogenic sources for energy and, depending upon the length of the fast, ketones. Gluconeogenesis becomes a very important source of glucose for fasting animals. For example, in rats this pathway has been shown to account for about 90% of their total glucose production after only two days of fasting compared to only about 50% when they are in the post-absorptive state (Peroni *et al.*, 1996; 1997). Glycerol, a product of triacylglycerol hydrolysis, accounts for approximately 21-26% of human and rat fasting gluconeogenesis glucose production (Baba *et al.*, 1995; Peroni *et al.*, 1997), and at least in humans, amino acids contribute an approximately equal amount to gluconeogenesis as glycerol (Owen *et al.*, 1998). The remaining glucose is derived from amino acids, lactate, pyruvate and acetone (Owen *et al.*, 1998). As stated earlier, the cells that are unable to oxidize circulating fatty acids can also use ketones as an alternative source of energy. Ketones are increasingly synthesized when the concentration of acetyl CoA exceeds the oxidative capacity of the liver. It is these high rates of gluconeogenesis, ketone formation and

ketone oxidation that occur in fasting animals that violate some of the assumptions of indirect calorimetry that were briefly discussed earlier in this chapter.

The nitrogenous waste products of amino acid oxidation are excreted in the urine as urea, ammonium ( $\text{NH}_4^+$ ), uric acid and creatinine, and often as a fast progresses, urinary urea nitrogen concentration drops and ammonia nitrogen becomes much more prominent (Owen *et al.*, 1998). In this study, urea concentration dropped in both the fasting woodchucks and rabbits, although only the woodchucks became more ammonotelic as the fast progressed. Interestingly, instead of increasing their excretion of ammonia, the rabbits increased their uric acid secretion. It is possible that the ability of fasting adapted animals (*e.g.* woodchucks) to decrease their rate of protein oxidation when fasting may act as a mechanism to reduce amino acid contribution to gluconeogenesis, thus forcing glucose production through other gluconeogenic pathways like glycerol, or a greater reliance on ketones (also a product of lipid metabolism). Conversely, they may just have an increased ability to use glycerol as a gluconeogenic precursor and, as a result, decrease protein contribution. Further research is needed to elucidate the exact mechanism that is used by these animals.

## **CHAPTER 3**

# **SUBSTRATE CYCLING, LIPOLYSIS AND FATTY ACID MOBILIZATION IN WOODCHUCKS FOLLOWING AROUSAL FROM HIBERNATION**

## **Introduction**

Like most hibernating animals, woodchucks do not remain in a continuous state of torpor until the spring, but rather go through periods of arousal and re-entry into hibernation throughout the winter. These pre- and post-torpor periods are very dynamic, as their body temperature and oxygen consumption change quickly (Lyman, 1958). These huge increases in metabolic rate and thermogenesis are produced by brown adipose tissue fatty acid oxidation, mitochondrial uncoupling of brown adipose tissue (non-shivering thermogenesis), and muscular shivering (Milner *et al.*, 1989; Nedergaard and Cannon, 1984). In fact, over ninety percent of the energy spent during hibernation has been estimated to occur during inter-torpor periods of arousal (Wang, 1978). Heat production by brown fat is thought to account for a high proportion of the heat that is produced during arousal (Foster, 1984). However, Lyman and O'Brien (1986) surprisingly showed that removal of 70-80% of brown adipose tissue from ground squirrels and hamsters did not disrupt their re-warming ability during arousal from hibernation. It has also been reported that removal of brown adipose tissue from a *Marmota* sp. has no immediate effect on their ability to arouse from hibernation (Dubois cited in Lyman and O'Brien, 1986). However, under these conditions it is not known if shivering or the remaining brown fat generates the heat that would have been provided by the excised tissue, or if other thermogenic mechanisms fill the deficit.

A possible mechanism hibernators may use for heat production during arousal and inter-torpor periods of hibernation that has not been investigated is thermogenesis through an increase in metabolic flux through specific substrate cycles. A substrate cycle occurs when concurrent flux through opposing reactions catalyzed by different non-equilibrium

enzymes uses energy without net conversion of substrate to product. Increased substrate cycling has been previously shown to be used for thermogenesis (Clark *et al.*, 1973b; Newsholme and Crabtree, 1976; Wolfe *et al.*, 1987a). One substrate cycle in particular is intrinsically part of triacylglycerol storage and breakdown. Triacylglycerol (TAG) hydrolysis (lipolysis) releases fatty acids (FA) to be used for either oxidative metabolism or reesterification (TAG resynthesis). Simultaneous lipolysis and reesterification form the TAG/FA cycle, a substrate cycle that dissipates fuel energy as heat (Newsholme and Crabtree, 1976; Wolfe *et al.*, 1987a). Consequently, I hypothesized that the TAG/FA substrate cycle may be an important source of endogenous heat for animals arousing from hibernation.

Quantification of the thermogenic contribution of this substrate cycle requires concurrent *in vivo* measurement of glycerol and fatty acid flux. Therefore, I planned on using the continuous infusion technique to simultaneously measure these parameters on woodchucks immediately before and during an arousal from hibernation (arousal can be easily initiated by the investigator). However, the continuous infusion technique requires the prior surgical insertion of two cannulas into the primary venous and arterial circulation of an anaesthetized animal. Unfortunately, the woodchucks did not reenter hibernation following the stimulation of this surgery so I was unable to perform continuous infusions on them while they were actively arousing from hibernation. Nevertheless, as the best possible alternative I measured their TAG/FA cycling rate two and eight days following their final arousal from hibernation. Therefore, any changes in glycerol and fatty acid flux and the production of endogenous heat by the TAG/FA substrate cycle that occur following arousal from hibernation would be detected.

## **Methods**

### *Hibernation and indirect calorimetry*

Near the end of September, woodchucks (male and female; n=8) were placed in large individual cages (1 m<sup>3</sup>) in a 5°C room with no light source. The cages were filled with nesting material (wood shavings, paper towels, paper, etc.) and a rural mailbox in which all of them eventually made a nest. Food was provided for five days and then only water. Within a few weeks, three of the woodchucks entered hibernation while the others did not. The extreme difficulty that I had getting the woodchucks to hibernate was the limiting factor for a low number of animals in the post-hibernation group. The woodchucks that did not hibernate were returned to their normal holding pens because they were rapidly losing weight. After a woodchuck had been hibernating for approximately one month, it was moved into a closed respirometer (54 x 38 x 67 cm) also filled with nesting material where it remained undisturbed for 2-4 weeks. Continuous 24 h measurements of hibernating woodchuck oxygen consumption and carbon dioxide production were recorded using an indirect calorimetry system (Columbus Instruments; Columbus, Ohio, USA) connected to the respirometer (described by Fournier and Weber, 1994). Every 24 h the oxygen and carbon dioxide sensors were calibrated using gases of known concentrations.

### *Surgery*

The woodchucks hibernated for a minimum of three months before they had cannulation surgery. The evening prior to surgery a hibernating woodchuck was manually disturbed to initiate an arousal from hibernation. At around midnight the animal was

placed in a room at 20 °C for the remainder of the night. At 7:00 the next morning the woodchuck was given a subcutaneous injection of buprenorphine (0.01 mg kg<sup>-1</sup>). One hour later, the animal was given an intra-muscular injection of ketamine (7 mg kg<sup>-1</sup>), midazolam (0.25 mg kg<sup>-1</sup>) and robinel (0.005 mg kg<sup>-1</sup>), placed under halothane/isoflurane anesthesia and intubated. Following sterile surgical criteria, I isolated the right jugular vein and right carotid artery and fed polyethylene (PE-50) saline-filled cannula into each vessel towards the heart (approximately 7 cm). Both cannulas were sutured to the vessels, the ends that were not in the vessels were tunneled under the skin, exteriorized through a small hole between the shoulder blades and sutured in place. The cannulas were filled with sterile saline containing heparin (40 units ml<sup>-1</sup>) and penicillin G (125 000 units ml<sup>-1</sup>), coiled and secured to the animal between its shoulder blades. The woodchuck was allowed to recover from the surgery at 22°C for a few hours and then was placed in the dark 5°C hibernation room in a closed respirometer (54 x 38 x 67 cm) filled with plenty of nesting material. No food was provided though water was freely available. This surgery was performed on the three woodchucks that had successfully entered hibernation in the fall. I opted not to flush their cannulas each day in the hope that they would reenter hibernation. Fortunately, the blood of the post-hibernation woodchucks had an extremely low clotting capacity and all their cannulas remained perfectly clear for the entire experimental period. Unfortunately, however, none of the woodchucks reentered hibernation.

Four additional non-hibernating woodchucks had cannulation surgery performed on them in mid-summer. Conversely, their blood had a very high clotting capacity, therefore, their cannulas were cleared, flushed with saline and re-filled with heparinized

saline each day. However, it was still extremely difficult to keep the cannulas patent for longer than a few days. In fact, two of the woodchucks died just a few days following surgery from thrombosis (determined by autopsy). Therefore, due to the limited success with the cannulas, high mortality and the expense of the woodchucks (> \$300 each), I opted not to conduct any more surgeries on normothermic woodchucks. The limiting factor for the small number of animals I had in the normothermic group was directly related to the problems associated with clotting of their blood following surgery.

### *Continuous infusion*

Two days following surgery, the cannulas of the woodchucks in the cold room were uncoiled and made accessible from outside the respirometer, and the lid was closed. Measurement of whole animal oxygen consumption and carbon dioxide production was started. Every 5 min until the end of the experiment, a 90 sec average of O<sub>2</sub> consumption and CO<sub>2</sub> production was recorded on a computer. The venous cannula was connected to a 10 cc syringe on a calibrated syringe pump (Harvard Apparatus; South Natick, MA, USA) set at 5 ml h<sup>-1</sup>. The syringe contained an infusate of trace amounts of 2-<sup>3</sup>H glycerol (5.39 ± 0.16 μCi ml<sup>-1</sup>) and 1-<sup>14</sup>C palmitate (7.84 ± 0.25 μCi ml<sup>-1</sup>). The infusate was prepared that day by mixing 1-[<sup>14</sup>C] palmitate (Amersham, Canada) with woodchuck plasma so the fatty acids could bind to albumin, adding 2-[<sup>3</sup>H] glycerol (Amersham, Canada) dissolved in saline (0.9% NaCl) and making the solution up to 5 ml with more saline. The radioactivity of each infusate was determined by counting it in ACS-II scintillation fluid (Amersham, Canada) using a Tri-Carb 2500 β-counter (Packard, Canada). Forty minutes after the start of the infusion the arterial cannula was used to take

a 1.5 ml blood samples every 5 min for 15 min. Immediately after sampling, the blood was centrifuged outside of the hibernation room and the plasma was separated and stored in glass containers at -20°C for analysis a few days later. Glass vials were used for storage as plastic easily absorbs glycerol. Measurement of indirect calorimetry was continued throughout the infusions. Throughout the experiments no noise was made and only a small red light (which the woodchucks could not detect) was used to light the room. Six days later a second continuous infusion was made. The animals were not disturbed between the first and second experiment.

The two non-hibernating woodchucks that kept their cannulas patent had two continuous infusions performed on them after they had been fasted for 2-3 days. This time period was actually optimal because in other fasting animals lipolytic rate has been shown to peak at this time (see Chapter 4). Therefore, I was able to compare fasting woodchuck lipid metabolism at normal temperatures to the lipid metabolism of woodchucks following hibernation. Data for the four infusions from these two woodchucks have been combined.

### *Analyses*

A sub-sample of the plasma was mixed with 25 ml of chloroform:methanol (Folch, 2:1) (Folch *et al.*, 1957) and centrifuged. Glass tubes and freshly distilled chloroform (BDH) stabilized with 0.75% ethanol were used (BDH). If old chloroform or chloroform stabilized with other stabilizers such as amylene is used, HCl can be produced in the chloroform causing a drop in pH (Georges Zwingelstein, personal communication; Université Claude Bernard, Lyon 1, France). This can cause degradation and modification of non-esterified fatty acids (NEFA) that have been shown to result in inaccurate

measurements (Patterson *et al.*, 1999). The aqueous phase (containing water soluble molecules; *e.g.* glycerol and glucose) and organic phase (containing water-insoluble molecules; *e.g.* lipids) were separated and similarly re-extracted with chloroform and methanol:water (40:30) respectively. The aqueous phase from each tube were pooled together as were the two organic phases. Each pooled phase was then dried using a rotating evaporator (Büchi RE 121 Rotavapor). The aqueous phase was resuspended in ethanol:water (1:1) and the organic phase in hexane:isopropanol (3:2). Glassware, pre-rinsed in Folch (2:1), was used throughout this extraction protocol to prevent absorption of glycerol into the walls of the tubes.

NEFA present in the organic phase were separated by liquid chromatography on Supelclean solid-phase extraction tubes (LC-NH<sub>2</sub>; Sigma, St. Louis, MO, USA). The NEFAs were extracted with isopropyl ether:acetic acid (98:2) after any neutral lipids had been eluted with chloroform:isopropanol (2:1). A sub-sample of the NEFA fraction was counted to determine the distribution of <sup>14</sup>C and <sup>3</sup>H activity in the lipids. Because <sup>14</sup>C activity is not incorporated in fatty acids other than palmitate, the activity found in the NEFA fraction is equal to palmitate activity. The remainder of the NEFA isopropyl ether:acetic acid fraction was used to determine the relative distribution of palmitate to the other fatty acids using a gas chromatograph (Hewlett-Packard 5890 series II) equipped with an automatic injection system (HP 7673) and flame-ionization detector.

NEFA methylation was performed using the method of Tserng *et al.* (1981). Briefly, the NEFA fraction was evaporated and methylated with 2,2-dimethoxypropane after adding concentrated HCl. The reaction was arrested about 1 hr later by adding pyridine and the NEFA methyl esters were recovered by two extractions with isooctane.

Analyses were performed on a 30 m fused silica capillary column (DB-23; J&W Scientific, Folsom, CA, USA) kept at 170 °C for 35 min after injection, raised to 220°C at a rate of 50°C min<sup>-1</sup>, and kept at that temperature for 50 min to burn off any remaining residue. The injection port temperature was 210°C and the detector was 250°C. Different NEFA standards were periodically injected for GC calibration. Heptadecanoic acid, a fatty acid found solely in plants, was used as an internal standard in the sample so fatty acid concentration could be quantified. This was done by adding a known amount of heptadecanoic acid to the organic phase of the initial extraction before the NEFAs were separated by liquid chromatography. Earlier experiments that compared NEFA concentrations determined using this GC method with those concentrations measured with an analytical test kit (NEFA C Wako Chemicals, Osaka, Japan) showed that loss of NEFA during the extraction protocol was small (< 5%).

A sub-sample of the aqueous phase was counted to determine its total <sup>3</sup>H activity (found in glycerol and glucose). Percentage activity in glycerol was obtained by separating glucose from glycerol using thin-layer chromatography (TLC). Briefly, a sample of the aqueous phase was evaporated and concentrated in a small amount of ethanol:water, this solution was then spotted on a silica gel plate (60 F<sub>254</sub>; Merk, Germany). The plate was developed using chloroform:methanol (8:3), and the separate glucose and glycerol sections scraped into separate scintillation vials, resuspended in ethanol:water (1:1) and counted. Glycerol concentration was also determined on a sub-sample of this phase. The solution was dried under nitrogen, resuspended in a hydrazine buffer, and its glycerol concentration measured with glycerol kinase and glycerol-phosphate dehydrogenase using a Beckman DU 640 spectrophotometer at 340nm (Weber

*et al.*, 1993). Earlier experiments with known amounts of labeled glycerol showed that storage and analysis of plasma in glassware reduced loss of glycerol to less than 2%.

Urine was collected from the summer woodchuck as described in Chapter 2, and total urinary nitrogen excretion was measured by the Kjeldahl method using Tecator Analysers (1007 Digester and Kjelttec System 1002 Distilling Unit). This method converts all the nitrogen in a sample into ammonium sulfate by digestion with a sulfuric acid mixture in the presence of sodium thiosulfate, potassium sulfate and copper sulfate. Excess alkali is then used to distill the ammonium sulfate and the free ammonia is collected in a known excess quantity of acid. This acid-ammonia mixture is then titrated with alkali to determine the ammonia concentration.

### *Calculations*

Lipid, carbohydrate and protein oxidation rates were calculated according to Frayn (1983):

$$\text{Glucose oxidation (g min}^{-1}\text{)} = 4.55 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 3.21 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 2.87 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

$$\text{Lipid oxidation (g min}^{-1}\text{)} = 1.67 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 1.67 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 1.92 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

The second equation assumes that all lipid oxidation is of palmitoyl-stearoyl-oleoyl-glycerol, the average fatty acid found in human adipose tissue. Assuming this is correct, oxidation rates can were then converted to their molar equivalent by using the molecular

weight of this triacylglycerol ( $861 \text{ g mol}^{-1}$ ) and glucose ( $180 \text{ g mol}^{-1}$ ). Finally, fatty acid oxidation was calculated by multiplying the molar rate of triacylglycerol oxidation by 3 as each mole of triacylglycerol contains 3 moles of fatty acids.

Total urinary nitrogen was used to estimate daily protein catabolism. A value of 6.25 g protein per g urine N was used to convert urinary N excretion to protein oxidation (Frayn, 1983). Urine could not be collected from the post-hibernation woodchucks because they were nesting, so their rate of urinary nitrogen excretion was estimated using the following information. Nitrogen excretion of hibernators in their aroused state does not differ from when they are fasted at normal temperatures (Galster and Morrison, 1975; Kastner *et al.*, 1978), and in Chapter 2 I showed that the nitrogen excretion rate of fasting woodchucks is constant after one week and does not differ with season, energy expenditure or body size. Therefore, I used the mean urinary N excretion rate of eight woodchucks during the last five days of a two week fast (40 observations). This gave me a value of  $1.71 \pm 0.31 \mu\text{mol N kg}^{-1} \text{ min}^{-1}$ .

The rates of appearance ( $R_a$ ) of glycerol and palmitate were calculated using the steady state equation of Steele (1959).

$R_a (\mu\text{mol kg}^{-1} \text{ min}^{-1}) = \text{isotope infusion rate (DPM kg}^{-1} \text{ min}^{-1}) / \text{plasma specific activity (SA; DPM } \mu\text{mol}^{-1}\text{)}$ ;

$\text{SA} = \text{plasma isotope activity (DPM ml}^{-1}\text{)} / \text{plasma metabolite concentration } (\mu\text{mol ml}^{-1}\text{)}$ ;

$\text{DPM} = \text{disintegrations per minute}$

Ra glycerol is assumed to be a direct measure of triacylglycerol hydrolysis as adipose tissue lacks glycerokinase and cannot metabolize glycerol, and incomplete hydrolysis of triacylglycerol into mono- and diacylglycerol is negligible (Arner and Ostman, 1974). This means that all the glycerol released by triacylglycerol hydrolysis should appear in the plasma. Ra NEFA was determined by dividing Ra palmitate by the fractional contribution of palmitate to total NEFA. The rate of total NEFA reesterification was calculated as the difference between 3 x Ra glycerol (total NEFA released from triacylglycerol) and the simultaneous rate of FA oxidation (see below). Steady-state conditions existed during blood sampling as changes in metabolite concentration, specific activity or rate of appearance were not observed over time. The samples for an individual were averaged following analysis. Rates of primary fatty acid reesterification (when fatty acids that are hydrolyzed from TAG are reesterified back into TAG without entering the circulation) and secondary reesterification (fatty acids that undergo lipolysis, enter the circulation and are then reesterified back into TAG in a different cell) were calculated according to Wolfe *et al.* (1990):

$$\text{Primary TAG:FA cycling} = (3 \times \text{Ra glycerol}) - \text{Ra NEFA},$$

$$\text{Total cycling} = (3 \times \text{Ra glycerol}) - \text{FA oxidation rate},$$

$$\text{Secondary TAG:FA cycling} = \text{total cycling} - \text{primary cycling}.$$

These calculations also assume that any fatty acids that are released by lipolysis will either be reesterified or move into the circulation. This is important because oxidation of fatty acids by adjacent tissue before movement into the plasma pool would result in an

underestimation of Ra NEFA. Fortunately, there is evidence to show that fatty acid oxidation by this route is probably small (Wolfe *et al.*, 1990). Any values of total TAG/FA recycling should be accurate as it does not depend upon either Ra NEFA or the site of fatty acid oxidation.

I also calculated the energetic cost of fatty acid reesterification knowing that the TAG/FA substrate cycle costs approximately  $603 \text{ kJ mol}^{-1}$  ( $144 \text{ kcal mol}^{-1}$ ) of recycled triacylglycerol (Wolfe *et al.*, 1990). This is because the TAG/FA cycle uses approximately eight high-energy phosphate bonds per mole of recycled triacylglycerol and about 75 kJ (18 kcal) of heat are released per mole of hydrolyzed ATP. Finally, oxygen consumption ( $\mu\text{mol kg}^{-1} \text{ min}^{-1}$ ) was converted to energy expenditure ( $\text{J kg}^{-1} \text{ min}^{-1}$ ; 0.45 J of heat per  $\mu\text{mol}$  of oxygen) so the contribution of this substrate cycle to the total energy expenditure of the animal could be calculated.

### *Statistics*

Statistical differences were tested for by using a one-way ANOVA and the Tukey post-hoc test. All data expressed as percentages were transformed to the arcsine of their square root before testing for differences (Zar, 1984). Statistical significance is indicated when  $p < 0.05$ . Results are presented as means  $\pm$  SEM.

### **Results**

The hibernating woodchucks were not in a constant state of deep hypothermia; rather they went through periodic arousal from hibernation. The woodchucks in this study aroused every  $150.5 \pm 4.5$  hours (average for 8 arousal periods). During such an arousal

their oxygen consumption increased by almost 100 fold within only a half-hour. Between arousals their hibernating oxygen consumption was  $11.2 \pm 1.81 \mu\text{mol kg}^{-1} \text{min}^{-1}$ . This increased to a peak of  $1053.0 \pm 57.3 \mu\text{mol kg}^{-1} \text{min}^{-1}$  during an arousal episode (Fig. 3.1). Following surgery the woodchucks did not re-enter hibernation, although they did remake their nests and when I was in the room with them they were curled up almost 100% of the time. Mean oxygen consumption during this time was  $185.3 \pm 4.70 \mu\text{mol kg}^{-1} \text{min}^{-1}$  (Fig. 3.2). This rate of oxygen consumption was not significantly different from those rates measured on separate groups of non-hibernating woodchucks in the spring and summer that had been fasting for two weeks,  $197.7 \pm 5.08 \mu\text{mol O}_2 \text{kg}^{-1} \text{min}^{-1}$  and  $203.2 \pm 20.5 \mu\text{mol O}_2 \text{kg}^{-1} \text{min}^{-1}$ , respectively (Fig. 3.2). The mean oxygen consumption of the 2-3 day fasted normothermic woodchucks used for the control continuous infusions was  $252.7 \pm 12.6 \mu\text{mol kg}^{-1} \text{min}^{-1}$ .

There was not a significant difference in plasma glycerol concentration between the post-hibernation woodchucks and the normothermic woodchucks (Fig. 3.3a), although the post-hibernation woodchucks did have a 56-108% significantly higher rate of lipolysis than the normothermic woodchucks ( $p < 0.05$ ; Fig. 3.3b). This difference depended upon how many days had passed since the woodchucks had been aroused from hibernation. At two days, the woodchuck's lipolytic rate was  $25.59 \pm 2.15 \mu\text{mol kg}^{-1} \text{min}^{-1}$ , compared to at eight days when their lipolytic rate was  $19.04 \pm 1.37 \mu\text{mol kg}^{-1} \text{min}^{-1}$ . This high rate of lipolysis did not occur with equally high rates of fatty acid movement into the circulation. While the rate of appearance of NEFA was 26% higher in the two day post-hibernation woodchucks compared to the normothermic woodchucks ( $p < 0.05$ ), there was no difference between the two at 8 days post-hibernation (Fig. 3.4b). No difference in NEFA

concentration was measured between the summer and post-hibernation woodchucks (Fig. 3.4a), although there were differences in size of an animal's adipose tissue depot relative to body mass ( $p < 0.05$ ; Table 3.1) and their plasma fatty acid composition ( $p < 0.05$ ; Table 3.2). Relative to total concentration, the saturated fatty acids either remained the same (palmitate; 16:0) or decreased (stearate; 18:0), while almost all the mono- and poly-unsaturated fatty acids increased. The exception was linolenate (18:3) that remained the same.

Fatty acid oxidation of the normothermic woodchucks was  $8.28 \pm 0.41 \mu\text{mol FA kg}^{-1} \text{ min}^{-1}$  (Fig. 3.5a), and contributed to about 90% of their total oxygen consumption. The contribution of lipids to the total oxygen consumption of the post-hibernation woodchucks was higher than the normothermic woodchucks (97% for both 2 and 8 day post-hibernation, respectively;  $p < 0.05$ ), however, their absolute rate of fatty acid oxidation was lower ( $6.76 \pm 0.32$  and  $6.86 \pm 0.21 \mu\text{mol FA kg}^{-1} \text{ min}^{-1}$  for 2 day and 8 day, respectively;  $p < 0.05$ ; Fig. 3.5a). When looking at fatty acid oxidation relative to the amount of fatty acids made available through triacylglycerol hydrolysis (lipolysis), an even greater difference is noted. The normothermic woodchucks oxidized 20% of the fatty acids while the post-hibernation woodchucks only oxidized 9-12% of them ( $p < 0.05$ ; Fig. 3.5b). The primary contributing factor to this difference was the 13-16% higher relative rate of fatty acid reesterification that was measured in the post-hibernation woodchucks compared to the normothermic woodchucks ( $p < 0.05$ ; Fig. 3.5b).

As a result, the total energetic requirement of the TAG/FA cycle in the normothermic woodchucks was approximately  $5.10 \pm 0.44 \text{ J kg}^{-1} \text{ min}^{-1}$ , compared to  $14.14 \pm 1.36$  and  $10.15 \pm 0.79 \text{ J kg}^{-1} \text{ min}^{-1}$  in the aroused woodchucks ( $p < 0.05$ ; Fig 3.6b).

The energy expenditure of the post-hibernation woodchucks was 27% lower than the energy expenditure of the normothermic woodchucks, therefore, when the cost of this substrate cycle is calculated relative to energy expenditure it accounts for 17.4% (2 day) and 12.0% (8 day) in the woodchucks that had been aroused from hibernation compared to only 4.46% in the normothermic woodchucks ( $p < 0.05$ ).

**Table 3.1. Body composition of a sub-sample of woodchucks as determined by carcass**

**dissection. Values are means  $\pm$  SEM and \* indicates a significant difference between the marked value and the value to the left. LBM = lean body mass.**

	<b>Spring (following hibernation)</b>	<b>Spring (no hibernation)</b>	<b>Summer</b>
<b>N</b>	<b>2</b>	<b>2</b>	<b>2</b>
<b>Body mass (kg)</b>	<b>2.593 <math>\pm</math> 0.007</b>	<b>2.828 <math>\pm</math> 0.372</b>	<b>3.955 <math>\pm</math> 0.200</b>
<b>Skeletal muscle (% LBM)</b>	<b>30.17 <math>\pm</math> 2.78</b>	<b>52.41 <math>\pm</math> 1.19 *</b>	<b>54.32 <math>\pm</math> 1.59</b>
<b>Adipose tissue (% body mass)</b>	<b>16.74 <math>\pm</math> 3.42</b>	<b>40.31 <math>\pm</math> 0.53 *</b>	<b>56.10 <math>\pm</math> 0.48 *</b>
<b>Heart (% LBM)</b>	<b>0.51 <math>\pm</math> 0.04</b>	<b>1.10 <math>\pm</math> 0.09 *</b>	<b>1.17 <math>\pm</math> 0.01</b>
<b>Liver (% LBM)</b>	<b>3.31 <math>\pm</math> 0.32</b>	<b>6.08 <math>\pm</math> 0.11 *</b>	<b>6.16 <math>\pm</math> 0.44</b>

**Table 3.2. Plasma composition of major fatty acids (% of total) in post-hibernation and 2-3 day fasted summer woodchucks. Values are means  $\pm$  SEM and \* indicates a significant difference between groups.**

<b>FATTY ACID</b>	<b>SUMMER</b>	<b>POST-HIBERNATION</b>
16:0 (palmitate)	26.4 $\pm$ 0.68	24.8 $\pm$ 1.01
18:0 (stearate)	37.7 $\pm$ 2.47	23.8 $\pm$ 1.71 * $\downarrow$
16:1 (palmitoleate)	0.90 $\pm$ 0.21	2.04 $\pm$ 0.37 * $\uparrow$
18:1 (oleate)	20.0 $\pm$ 1.96	28.2 $\pm$ 2.47 * $\uparrow$
18:2 (linoleate)	13.3 $\pm$ 0.90	19.3 $\pm$ 1.36 * $\uparrow$
18:3 (linolenate)	1.70 $\pm$ 0.45	1.90 $\pm$ 0.51

**Figure 3.1. Woodchuck oxygen consumption ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) during arousal from and subsequent re-entry into hibernation. The values are means  $\pm$  SEM for two woodchucks that each had four arousal episodes. The dotted line is an indicator of the mean oxygen consumption of the woodchucks during the post-hibernation period. The graph insert is an expansion of the peak.**

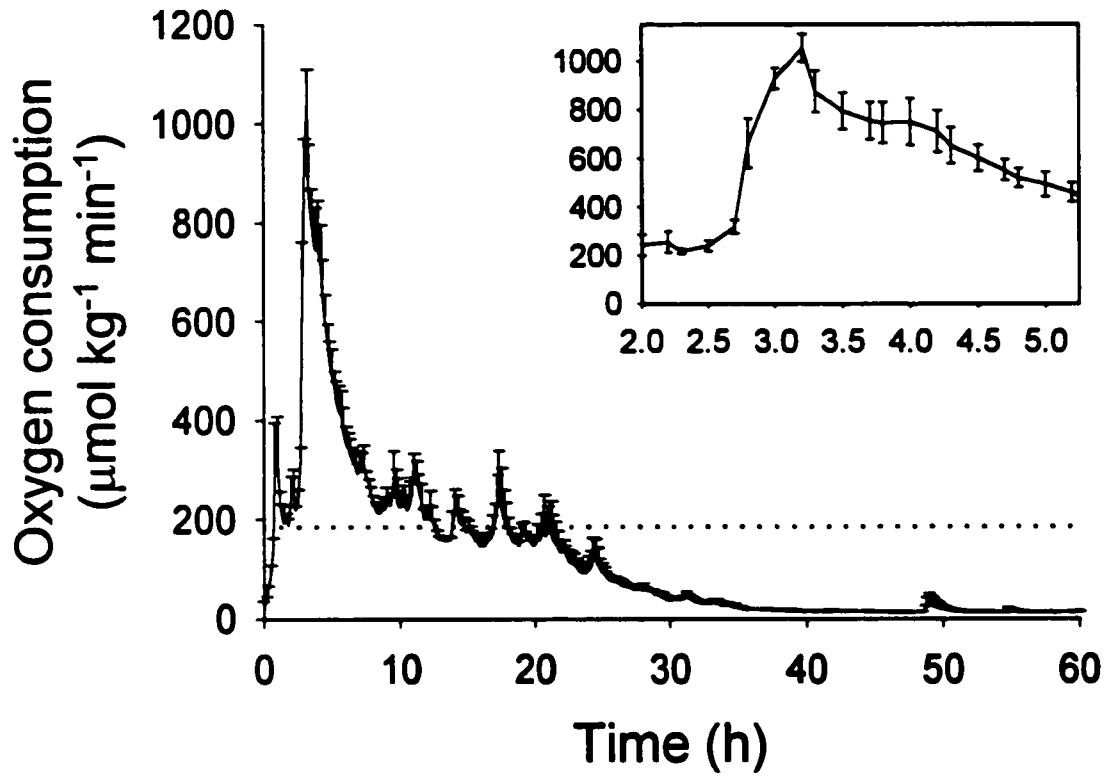


Figure 3.1

**Figure 3.2. a) Mean twenty-four hour oxygen consumption ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) of normothermic and hypothermic woodchucks under different fasting conditions, with the oxygen consumption of fed woodchucks added for comparison. Values are means  $\pm$  SEM and \* indicates a significant difference from all.**

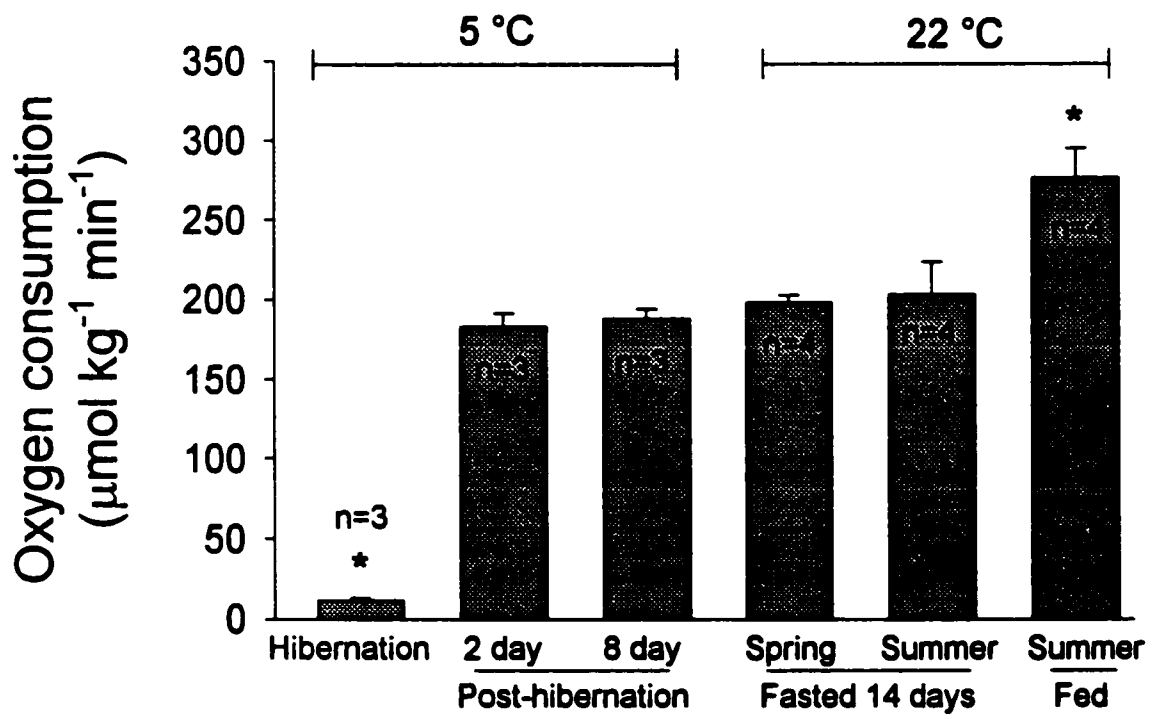


Figure 3.2

**Figure 3.3. a) Plasma glycerol concentration ( $\mu\text{M}$ ) and b) glycerol rate of appearance or lipolysis ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) of normothermic (n=4) and post hibernation (n=3) woodchucks. Values marked with the same symbol are significantly different from each other. Values are means  $\pm$  SEM.**

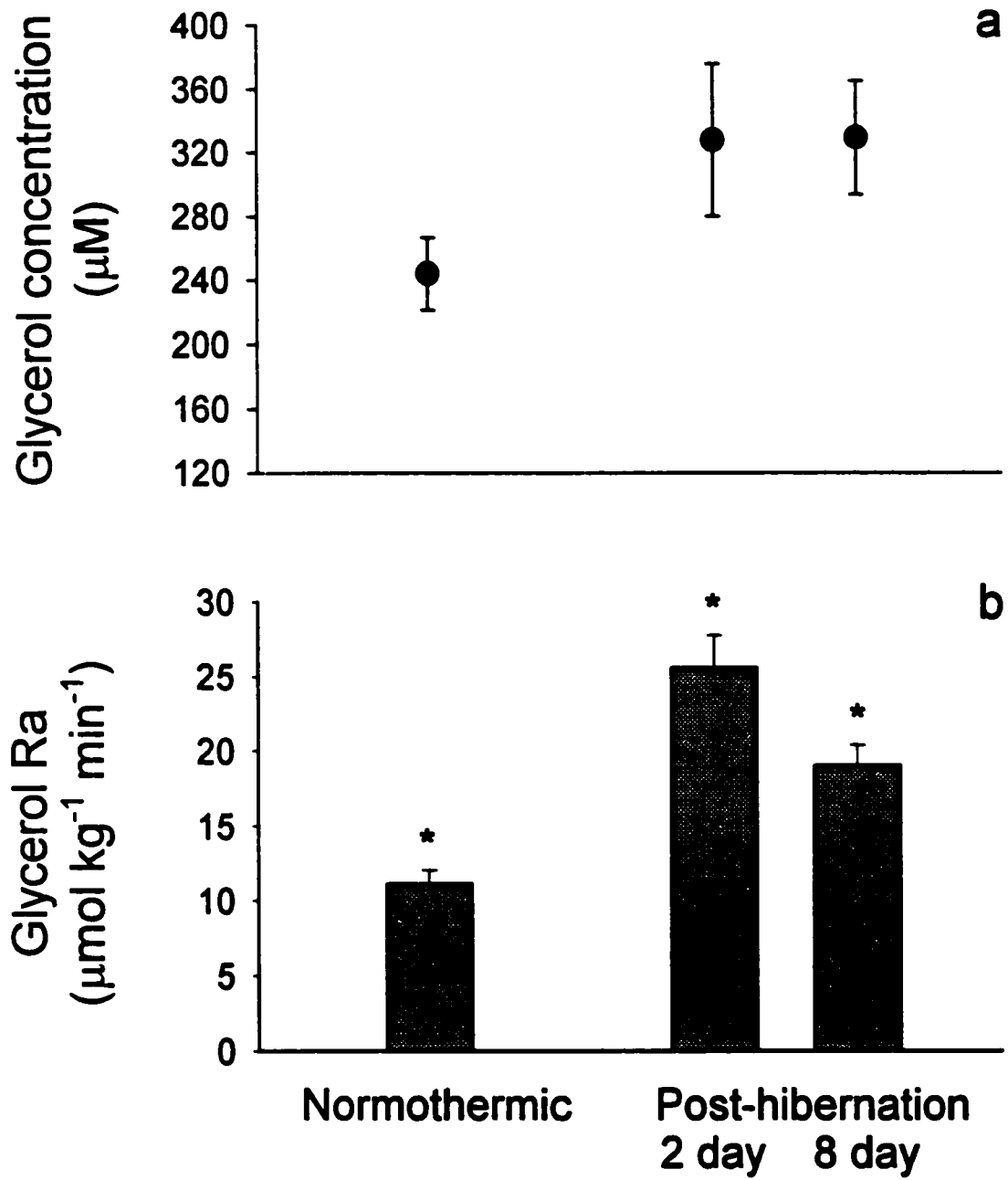


Figure 3.3

**Figure 3.4. a) Plasma non-esterified fatty acid (NEFA) concentration (mM) and b) NEFA rate of appearance ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) of normothermic (n=4) and post hibernation (n=3) woodchucks. Values marked with the same symbol are significantly different from each other. Values are means  $\pm$  SEM.**

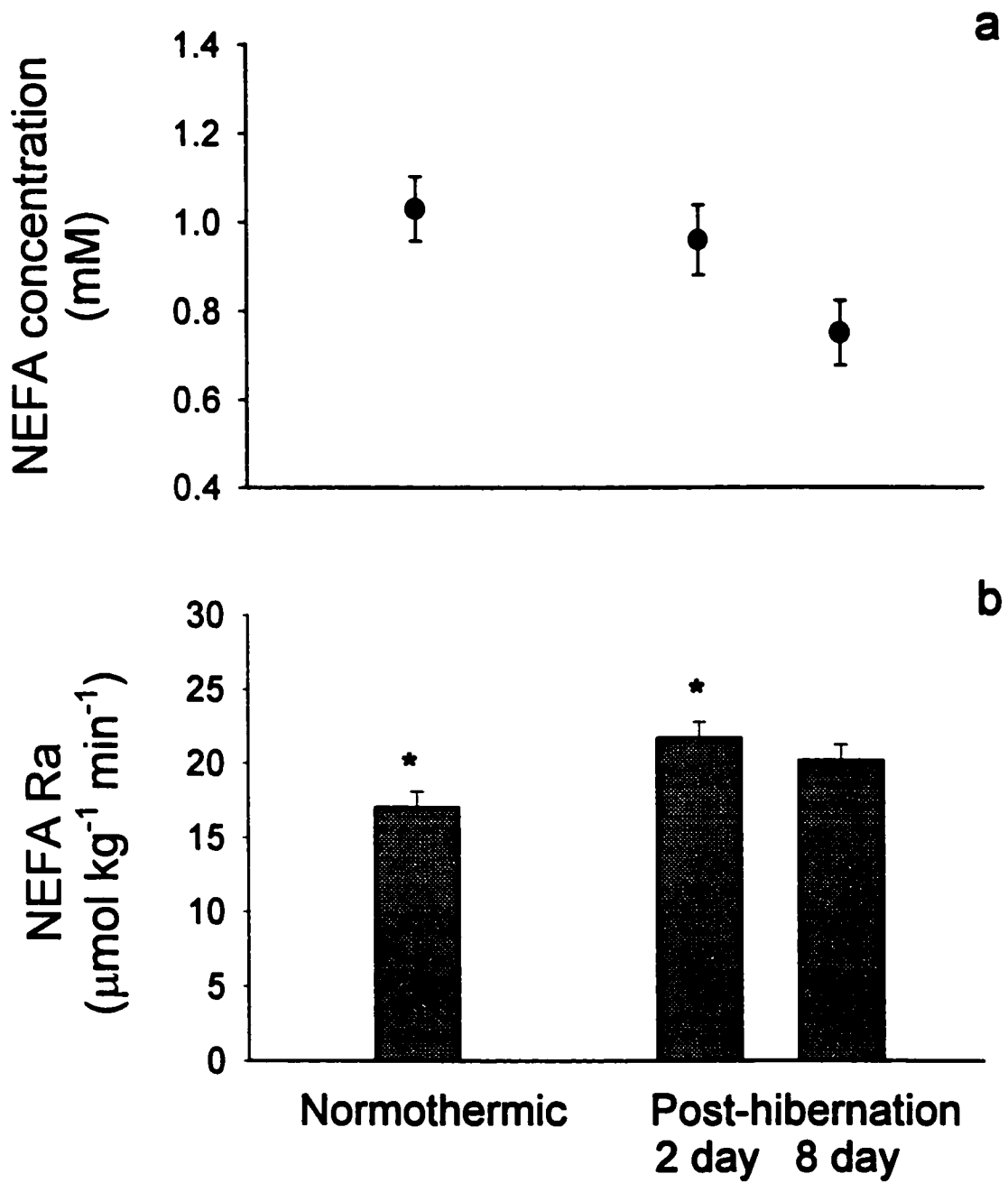


Figure 3.4

**Figure 3.5. Rate of fatty acid oxidation (dark bars) and reesterification (light bars) expressed in a) absolute values ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) and b) relative to the total amount of fatty acids released by lipolysis from triacylglycerol. Values marked with the same symbol are significantly different from each other. Values are means  $\pm$  SEM.**

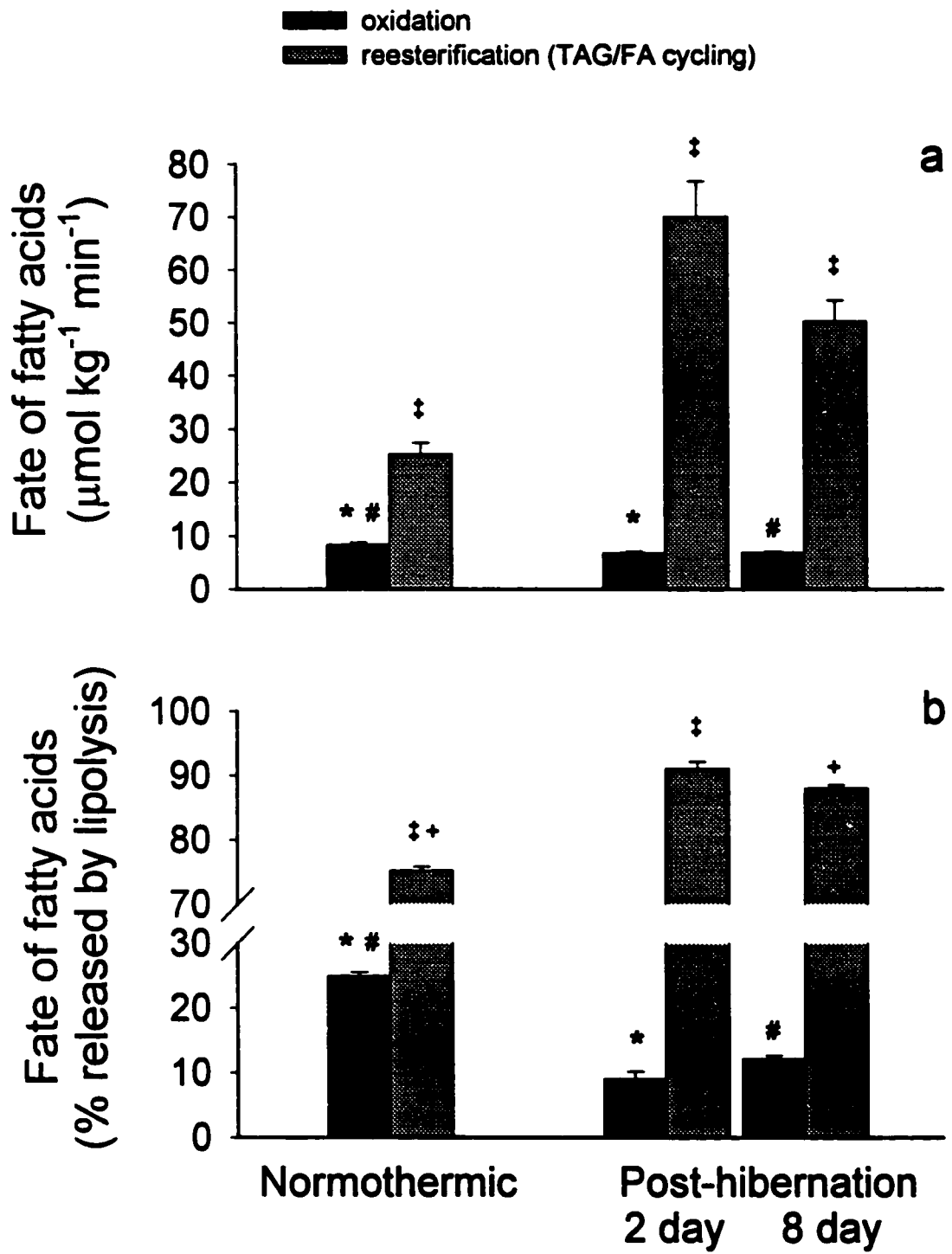


Figure 3.5

Figure 3.6. a) Total aerobic energy expenditure of normothermic (n=4) and post hibernation (n=3) woodchucks expressed in joules of heat per kg per min ( $\text{J kg}^{-1} \text{min}^{-1}$ ), b) the energetic cost of the TAG/FA substrate cycle also expressed in absolute values ( $\text{J kg}^{-1} \text{min}^{-1}$ ) and c) the energetic cost of the TAG/FA substrate cycle expressed relative to the woodchucks total energy expenditure. Values marked with the same symbol are significantly different from each other. Values are means  $\pm$  SEM.

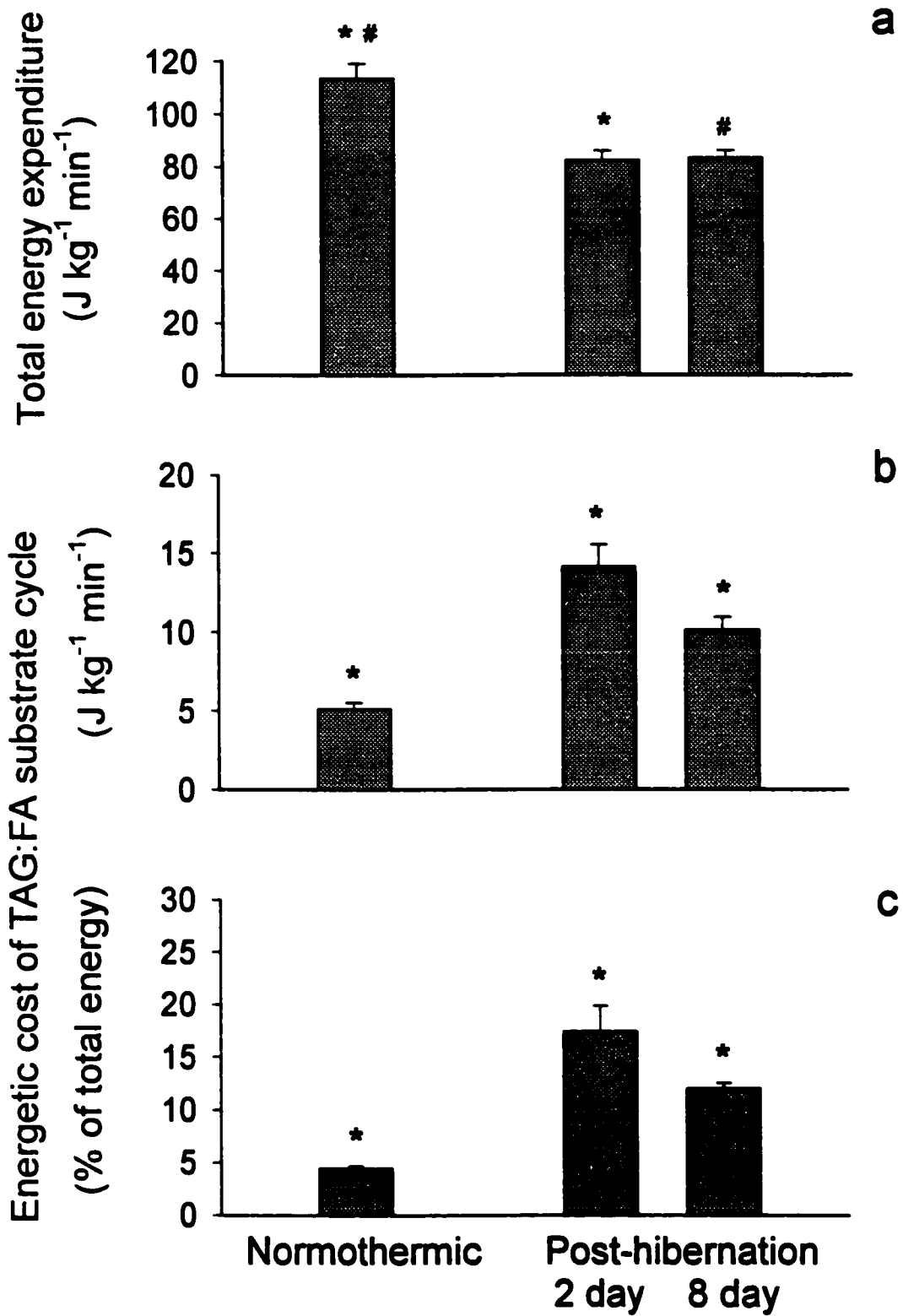


Figure 3.6

## **Discussion**

Many substrate cycles have been experimentally shown to significantly contribute to heat production in mammals as well as insects (Clark *et al.*, 1973b; Newsholme, 1978; Newsholme and Crabtree, 1976; Wolfe *et al.*, 1987a). The thermogenic potential of these cycles is so great that if they cycle out of control, they can actually create so much internal heat that tissue damage or even death can result. For example, when some humans and a strain of pigs carrying a particular gene mutation are exposed to certain inhaled anesthetics they can have uncontrolled thermogenesis, or deadly malignant-hyperthermia (Denborough, 1998). In pigs, some of that heat that is generated is from a dramatic increase in the cycling rate of the fructose 6-phosphate/fructose bisphosphate substrate cycle (Clark *et al.*, 1973b). The most well studied substrate cycle to date has been the TAG/FA cycle. This cycle usually accounts for only a small percentage of total resting energy expenditure (0.4-2.3% in humans (Bahr *et al.*, 1990; Elia *et al.*, 1987; Wolfe *et al.*, 1987a; Wolfe *et al.*, 1990; Wolfe and Peters, 1987); 2.9% in rats (McClelland *et al.*, 2000); 3.2% in rabbits (Chapter 4)).

In this study, the woodchucks that were at 5°C and had just aroused two days earlier from hibernation had an increased rate of TAG/FA cycling that was 178% higher than what was measured in woodchucks at 22°C that had been fasting for 2-3 days. The rate of fatty acid reesterification in the normothermic woodchucks was probably elevated as well, as short-term fasting has previously been shown to cause an increase in the TAG/FA cycling rate of humans (Elia *et al.*, 1987; Wolfe *et al.*, 1987b) and rabbits (Chapter 4). The high rate of substrate cycling that was measured in the newly aroused woodchucks actually accounted for about 17% of their total energy expenditure. This is

the largest contribution to metabolic rate by this substrate cycle that has ever been measured. The result of such a high rate of fatty acid reesterification can also be seen in how close the rate of appearance of NEFAs is to the rate of appearance of glycerol. Usually, fasting animals have Ra NEFA/Ra glycerol ratios around 2 (the theoretical maximum is 3) as many of their fatty acids are mobilized along with the glycerol so that they can be used for  $\beta$ -oxidation. The normothermic fasting woodchucks had a Ra NEFA/Ra glycerol of 1.5, however, the 2 day post-hibernation woodchucks had a Ra NEFA/Ra glycerol ratio that was only 0.85.

An obvious hypothesis to explain such high cycling rates of this substrate cycle is that the post-hibernation woodchucks are using it to generate heat now that they are again homeothermic and being maintained in a cold environment. One recent study using humans has looked at the effects of cold exposure (also at 5°C) on fatty acid reesterification rates (Vallerand *et al.*, 1999). Under cold exposure, the rate of fatty acid reesterification increased by almost 200% compared to the reesterification rates of thermoneutral controls. This occurred with a 2.7 times increase in total energy expenditure, therefore, when the cost of this substrate cycle was calculated relative to metabolic rate its contribution was approximately the same. The substrate cycle contributed about 3% to the total energy expenditure of the subjects, regardless of environmental temperature. Therefore, Vallerand *et al.* (1999) showed that humans exposed to cold are able to use this substrate cycle as a warming mechanism. The decrease in woodchuck fatty acid reesterification (absolute values and relative to energy expenditure) with time following arousal from hibernation (even though the room temperature remained at 5°C) leads one to doubt that the high thermogenic contribution of

the TAG/FA substrate cycle to the woodchucks was entirely related to environmental temperature. The higher contribution of the substrate cycle to energy expenditure at two days vs. eight days post-hibernation actually leads one to speculate that the TAG/FA cycling rate was declining from some sort of peak, which possibly occurred at the time the animals were arousing from hibernation.

As seen with the woodchucks in this study, a huge amount of energy is used during arousal from hibernation mainly as a mechanism to increase their core body temperature within a very short period of time so that their normal body function can return (Lyman, 1958). Hibernators use a number of different mechanisms to increase their body temperature, and essentially all are designed to waste energy while producing heat (*e.g.* mitochondrial uncoupling in brown fat). Substrate cycles fall into this category and the results of this study show that the TAG/FA substrate cycle accounts for a high 17%, and decreasing, proportion of the energy expended by a hibernator within the days following arousal from hibernation. Therefore, this substrate cycle, as well as others, may contribute a large proportion of the metabolic heat that is produced by hibernators during arousal from hibernation throughout the winter season.

## **CHAPTER 4**

# **TEMPORAL CHANGES IN RABBIT SUBSTRATE CYCLING, LIPOLYSIS, AND FATTY ACID MOBILIZATION DURING LONG- TERM FASTING**

## **Introduction**

**Energy storage in our bodies occurs almost exclusively as triacylglycerol.**

**Lipolysis is the process that releases fatty acids from our stored triacylglycerol so that they can be mobilized to other sites where they may be oxidized to provide us with energy in a form that we can use (ATP). Reesterification back into triacylglycerol is the only other possible fate for these released fatty acids. Fatty acid reesterification can occur both in the cell where lipolysis occurred and in a different cell if the fatty acid moves into the circulation (Campbell *et al.*, 1992; Wolfe *et al.*, 1990). Lipolysis and reesterification is a continuous process in adipocytes, and this cycle of triacylglycerol hydrolysis into fatty acids and glycerol and then reesterification back into triacylglycerol is called the triacylglycerol/fatty acid (TAG/FA) substrate cycle. A substrate cycle exists when concurrent flux through opposing reactions are catalyzed by different enzymes and use energy without net conversion of substrate to product (Newsholme and Crabtree, 1976).**

**Short-term fasting in humans, where weight loss was less than 5%, has been shown to cause significant increases in the rate of appearance (Ra) of glycerol, an indicator of lipolytic rate, and fatty acid mobilization (Klein *et al.*, 1986; 1993; Wolfe *et al.*, 1987b). It is unknown, however, if these rates of lipolysis and fatty acid mobilization change during more prolonged fasting. Plasma glycerol and fatty acid concentrations in rats decrease with fasting time (Parrilla, 1978). This suggests that the high rate of appearance of fatty acids measured at the beginning of a fast may be transient and actually decrease with fasting time. Short-term fasting in humans has also been shown to cause increases in fatty acid reesterification (TAG/FA substrate cycling) (Elia *et al.*, 1987; Wolfe *et al.*, 1987b).**

Substrate cycles have been shown by investigators to contribute to increases in energy expenditure under many different physiological conditions (Bahr *et al.*, 1990; Clark *et al.*, 1973a; Clark *et al.*, 1973b; Romijn *et al.*, 1993; Vallerand *et al.*, 1999; Wolfe *et al.*, 1987a; Wolfe *et al.*, 1990). Therefore, an increase in substrate cycling with fasting is puzzling because this is a physiological state that requires an individual to conserve energy for prolonged survival. In fact, most mammals do depress their metabolism when fasted, even those that are not fasting adapted (Keys *et al.*, 1950; Merkt and Taylor, 1994). A reduction in energy expenditure with fasting serves to lower ATP demand, use of metabolic fuels and possibly, lipolytic rates and fatty acids mobilization. Therefore, the objective of this study was to measure any temporal changes in TAG/FA substrate cycling, lipolysis and fatty acid mobilization that occur during long-term fasting (> 5% weight loss), and to discuss them in relation to any changes that may also occur in energy expenditure.

## **Methods**

### *Animals and surgery*

Six New Zealand white rabbits (3 male and 3 female) weighing  $2.96 \pm 0.11$  kg were used in this study. Daily food and water intake was recorded for each rabbit for 3 days prior to surgery and each day following. They were fed rabbit chow (52% carbohydrate, 3% fat, 16% protein, 14% crude fiber, 15% moisture). On the morning of surgery, the rabbits were pre-medicated with an intra-muscular injection of ketamine (15 mg kg<sup>-1</sup>), midazolam (0.5 mg kg<sup>-1</sup>) and robinel (0.005 mg kg<sup>-1</sup>), placed under halothane/isoflurane anesthesia with oxygen and intubated. Using sterile surgical technique I isolated the right jugular vein and right carotid artery and fed into each vessel

saline filled PE-50 cannula approximately 7 cm towards the heart. Both cannulas were sutured to the vessels, tunneled under the skin, exteriorized through a small hole between the shoulder blades and sutured in place. The cannulas were filled with sterile saline containing heparin (40 units ml<sup>-1</sup>) and penicillin G (125 000 units ml<sup>-1</sup>), coiled and secured to the animal between its shoulder blades. The rabbits were given buprenorphine (0.02 mg kg<sup>-1</sup> subcutaneous) on the morning and afternoon of the day of surgery and 1 day following to eliminate post-operative pain. To keep the cannulas patent, the heparinized saline was withdrawn each day, flushed with saline and re-filled with heparinized saline. Because heparin has lipolytic properties the heparinized saline was always removed from the cannulas before any solution was flushed into the animal. The rabbits were used for an experiment 3-5 days following surgery and always after food and water consumption had been at pre-operative levels for at least 2 days.

### *Experimental Design*

A single rabbit was contained in a closed Plexiglas respirometer (54 x 38 x 67 cm) supplied with room air at 3-8 l min<sup>-1</sup> depending upon the animal's metabolic rate. A small enclosed fan in the respirometer lid circulated the air inside the chamber. For the first 3 days the rabbit was given free access to food and water and then only water was provided. This continued until either 6 days had passed or the rabbit lost 15% of its initial body mass. Continuous 24 h measurements of whole animal oxygen consumption and carbon dioxide production were recorded using an indirect calorimetry system (Columbus Instruments; Columbus, Ohio, USA) connected to the respirometer (described by Fournier and Weber, 1994). Twenty-four hour urine samples were collected as it passed through

the metal floor of the respirometer and funneled into an attached polyethylene bottle kept on ice. To help prevent bacterial growth, a crystal of thymol was added to the bottle before urine collection was started for the day.

Every 24 h the respirometer was cleaned, and the oxygen and carbon dioxide sensors were calibrated using gases of known concentrations. As well, each cannula was flushed, water and food consumption, feces and urinary excretion, and the weight and body temperature of the rabbit were recorded. On the third day following placement of the rabbit in the respirometer, and every second day following removal of food, a continuous infusion was performed on the animal. One rabbit lost 15% of its body mass before 6 days had passed, so  $n=6$  for all time periods except for day 6 when  $n=5$ .

#### *Continuous infusion*

The respirometer was temporarily opened and the cannulas were uncoiled and made accessible from outside. The venous cannula was connected to a 10 cc syringe on a calibrated syringe pump (Harvard Apparatus; South Natick, MA, USA) set at  $5 \text{ ml h}^{-1}$ . The syringe contained an infusate of trace amounts of  $2\text{-}^3\text{H}$  glycerol ( $7.90 \pm 0.32 \mu\text{Ci ml}^{-1}$ ) and  $1\text{-}^{14}\text{C}$  palmitate ( $8.39 \pm 0.55 \mu\text{Ci ml}^{-1}$ ). The infusate was prepared daily by mixing  $1\text{-}^{14}\text{C}$  palmitate with rabbit albumin dissolved in saline (0.9% NaCl), adding  $2\text{-}^3\text{H}$  glycerol and making the solution up to 5 ml with saline. The radioactivity of each infusate was determined by counting it on a Tri-Carb 2500  $\beta$ -counter (Packard, Canada). Forty minutes after the start of the infusion the arterial cannula was used to take a 1.5 ml blood sample every 5 min for 15 min. Immediately after sampling, the blood was centrifuged and the plasma was separated and stored in glass containers on ice until the end of the

experiment when it was frozen at -20 °C for analysis a few days later. Measurement of indirect calorimetry was continued throughout the infusion.

### *Analyses*

Details for plasma analysis are given in Chapter 3. In brief, total and individual plasma fatty acid concentration were measured by capillary GC analysis while glycerol concentration was measured enzymatically. Palmitate and glycerol activities were quantified by scintillation counting following separation by ion-exchange column and TLC, respectively. Steady-state conditions existed during blood sampling as changes in metabolite concentration, specific activity or rate of appearance were not observed over time. Therefore, measurements for each individual were averaged following analysis. Total urinary nitrogen excretion was measured by the Kjeldahl method.

### *Calculations*

Lipid, carbohydrate and protein oxidation rates were calculated according to Frayn (1983).

$$\text{Glucose oxidation (g min}^{-1}\text{)} = 4.55 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 3.21 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 2.87 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

$$\text{Lipid oxidation (g min}^{-1}\text{)} = 1.67 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 1.67 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 1.92 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

The second equation assumes that all lipid oxidation is of palmitoyl-stearoyl-oleoyl-glycerol, the average fatty acid found in human adipose tissue. Assuming this is correct, oxidation rates can be then converted to their molar equivalent by using the molecular weight of this triacylglycerol ( $861 \text{ g mol}^{-1}$ ) and glucose ( $180 \text{ g mol}^{-1}$ ). Finally, fatty acid oxidation was calculated by multiplying the molar rate of triacylglycerol oxidation by 3 as each mole of triacylglycerol contains 3 moles of fatty acids.

The rates of appearance ( $R_a$ ) of glycerol and palmitate were calculated using the steady state equation of Steele (1959).

$R_a (\mu\text{mol kg}^{-1} \text{ min}^{-1}) = \text{isotope infusion rate (DPM kg}^{-1} \text{ min}^{-1}) / \text{plasma specific activity (SA; DPM } \mu\text{mol}^{-1}\text{)}$ ;

$\text{SA} = \text{plasma isotope activity (DPM ml}^{-1}\text{)} / \text{plasma metabolite concentration } (\mu\text{mol ml}^{-1}\text{)}$ ;

DPM= disintegrations per minute

$R_a$  glycerol is assumed to be a direct measure of triacylglycerol hydrolysis as adipose tissue lacks glycerokinase and cannot metabolize glycerol, and incomplete hydrolysis of triacylglycerol into mono- and diacylglycerol is negligible (Arner and Ostman, 1974). This means that all the glycerol released by triacylglycerol hydrolysis should appear in the plasma.  $R_a$  NEFA was determined by dividing  $R_a$  palmitate by the fractional contribution of palmitate to total NEFA. The rate of total NEFA reesterification was calculated as the difference between  $3 \times R_a$  glycerol (total NEFA released from triacylglycerol) and the simultaneous rate of FA oxidation (see below).

Steady-state conditions existed during blood sampling as changes in metabolite concentration, specific activity or rate of appearance were not observed over time. The samples for an individual were averaged following analysis. Rates of primary fatty acid reesterification (when fatty acids that are hydrolyzed from TAG are reesterified back into TAG without entering the circulation) and secondary reesterification (fatty acids that undergo lipolysis, enter the circulation and are then reesterified back into TAG in a different cell) were calculated according to Wolfe *et al.* (1990):

$$\text{Primary TAG:FA cycling} = (3 \times \text{Ra glycerol}) - \text{Ra NEFA},$$

$$\text{Total cycling} = (3 \times \text{Ra glycerol}) - \text{FA oxidation rate},$$

$$\text{Secondary TAG:FA cycling} = \text{total cycling} - \text{primary cycling}.$$

These calculations also assume that any fatty acids that are released by lipolysis will either be reesterified or move into the circulation. This is important because oxidation of fatty acids by adjacent tissue before movement into the plasma pool would result in an underestimation of Ra NEFA. Fortunately, there is evidence to show that fatty acid oxidation by this route is probably small (Wolfe *et al.*, 1990). Any values of total TAG/FA recycling should be accurate as it does not depend upon either Ra NEFA or the site of fatty acid oxidation.

I also calculated the energetic cost of fatty acid reesterification knowing that the TAG/FA substrate cycle costs approximately  $603 \text{ kJ mol}^{-1}$  ( $144 \text{ kcal mol}^{-1}$ ) of recycled triglyceride (Wolfe *et al.*, 1990). This is because the TAG/FA cycle uses approximately eight high-energy phosphate bonds per mole of recycled triacylglycerol and about 75 kJ

(18 kcal) of heat are released per mole of hydrolyzed ATP. Finally, oxygen consumption ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) was converted to energy expenditure ( $\text{J kg}^{-1} \text{min}^{-1}$ ; 0.45 J of heat per  $\mu\text{mol}$  of oxygen) so the contribution of this substrate cycle to the total energy expenditure of the animal could be calculated.

### *Statistics*

Statistical differences were tested for by using a repeated measures ANOVA and the Bonferroni post-hoc test. All data expressed as percentages were transformed to the arcsine of their square root before testing for differences (Zar, 1984). Statistical significance is indicated when  $p < 0.05$ . Results are presented as means  $\pm$  SEM.

### **Results**

Rabbit mass decreased about 16% during the 6 day fast ( $p < 0.05$ ; Fig. 4.1). This difference in weight loss was probably due primarily to a change in the size of their adipose tissue. Carcass analysis of a sub-sample of fed and 7 day fasted rabbits showed that fasted rabbits had approximately four times less adipose tissue relative to body mass than fed rabbits ( $p < 0.05$ ; Table 4.1).

There was also a gradual decrease in aerobic energy expenditure of the rabbits as twenty-four hour oxygen consumption dropped 28% over the six day period ( $p < 0.05$ ; Fig. 4.2). Fasting initiated a 59% increase in lipolytic rate ( $p < 0.05$ ; Fig. 4.3b). Glycerol flux (Ra) increased from  $9.62 \pm 0.72$  to  $15.29 \pm 0.96 \mu\text{mol kg}^{-1} \text{min}^{-1}$  by day 2 of the fast ( $p < 0.05$ ), however, by day 4 the rabbit's lipolytic rate had dropped back down to fed levels. These changes in lipolytic rate were mimicked by changes in plasma glycerol

concentration ( $p < 0.05$ ; Fig. 4.3a). The increase in lipolytic rate at the beginning of the fast also resulted in a 73% higher rate of appearance of NEFA into the circulation ( $18.05 \pm 2.55$  vs.  $31.25 \pm 1.93 \mu\text{mol kg}^{-1}\text{min}^{-1}$ ;  $p < 0.05$ ; Fig. 4.4b). The plasma composition of fatty acids (% of total) also changed during the fast (Table 4.2). The contribution of stearate (18:0) to the plasma fatty acid pool dropped by 7.2%, while the contributions of oleate (18:1) and linoleate (18:2) increased by 4.3 and 5.6%, respectively. By the end of the fast the rate of appearance of NEFA had dropped back down to the level that was measured prior to the fast. This increase in NEFA flux ( $R_a$ ) also resulted in a transient increase in plasma NEFA concentration ( $p < 0.05$ ; Fig. 4.4a), which dropped back to fed levels at day 6.

The percentage of fatty acids made available by TAG hydrolysis ( $3 \times R_a$  glycerol) that were reesterified back into TAG dropped from 88% when the animals were being fed, to 67% by the end of the 6 day fast ( $p < 0.05$ ; Fig. 4.5b). This decrease in fatty acid reesterification occurred with a concurrent increase in the percentage of fatty acids being fed through the  $\beta$ -oxidation pathway. Fatty acid oxidation, expressed as a percentage of fatty acids made available by lipolysis, increased from 12% when fed to 33% by the end of the fast ( $p < 0.05$ ; Fig. 4.5b). In absolute values, fatty acid oxidation (as measured during the infusions) increased from  $3.48 \pm 0.28$  to a maximum of  $8.54 \pm 1.06 \mu\text{mol kg}^{-1}\text{min}^{-1}$  during the fast, an increase of 145% ( $p < 0.05$ ; Fig. 4.5a).

When the rates of fatty acid reesterification are subdivided into primary and secondary reesterification, about 47% of the released fatty acids were reesterified through secondary reesterification and 30% via primary reesterification (Fig. 4.6). The energetic cost of this cycle was higher on day 2 of the fast compared to the pre-fasting level ( $7.55 \pm$

0.59 vs.  $5.13 \pm 0.41 \text{ J kg}^{-1}\text{min}^{-1}$ ;  $p < 0.05$ ), although following this increase it dropped to below post-absorptive levels ( $p < 0.05$ ; Fig. 4.7a). This transient increase in fatty acid reesterification even caused an increase in its thermogenic contribution to the total energy expenditure of the animals ( $p < 0.05$ ; Fig. 4.7b).

**Table 4.1. Body composition of a sub-sample of fed and 6 day fasted rabbits. LBM = lean body mass. Values are means  $\pm$  SEM and \* indicates a significant difference between the fed and fasted value.**

	<b>FED</b>	<b>FASTED</b>
<b>N</b>	<b>3</b>	<b>3</b>
<b>Body mass (kg)</b>	<b>3.633 <math>\pm</math> 0.088</b>	<b>2.624 <math>\pm</math> 0.063</b>
<b>Skeletal muscle (% LBM)</b>	<b>48.23 <math>\pm</math> 0.70</b>	<b>43.09 <math>\pm</math> 2.42 *</b>
<b>Adipose tissue (% body mass)</b>	<b>17.85 <math>\pm</math> 1.80</b>	<b>4.11 <math>\pm</math> 0.08 *</b>
<b>Heart (% LBM)</b>	<b>0.38 <math>\pm</math> 0.02</b>	<b>0.27 <math>\pm</math> 0.04 *</b>
<b>Liver (% LBM)</b>	<b>3.21 <math>\pm</math> 0.27</b>	<b>2.00 <math>\pm</math> 0.32 *</b>

Table 4.2. Changes in the plasma composition of fatty acids (% of total) during six days of fasting. Values are means  $\pm$  SEM, n= 6 for all times except day 6 when n=5 and \* indicates a significant difference from the fed value.

Days fasted	16:0	18:0	16:1	18:1	18:2	18:3
0	28.3 $\pm$ 1.64	31.1 $\pm$ 2.47	1.99 $\pm$ 0.43	13.9 $\pm$ 0.86	12.7 $\pm$ 0.93	7.09 $\pm$ 2.22
2	28.5 $\pm$ 1.38	21.6 $\pm$ 2.37*	3.02 $\pm$ 0.67	19.3 $\pm$ 1.19*	19.8 $\pm$ 2.07*	6.07 $\pm$ 1.08
4	28.2 $\pm$ 2.15	24.2 $\pm$ 2.37*	2.11 $\pm$ 0.18	18.0 $\pm$ 0.79*	16.3 $\pm$ 1.62*	7.04 $\pm$ 1.77
6	30.5 $\pm$ 1.07	23.9 $\pm$ 2.38*	2.01 $\pm$ 0.36	18.2 $\pm$ 1.15*	18.3 $\pm$ 1.50*	4.05 $\pm$ 0.96
Change	—	↓	—	↑	↑	—

**Figure 4.1. Body mass of rabbits before (time=0) and during a six day fast. N=6 for all times except day 6 when n=5. Values are means  $\pm$  SEM.**

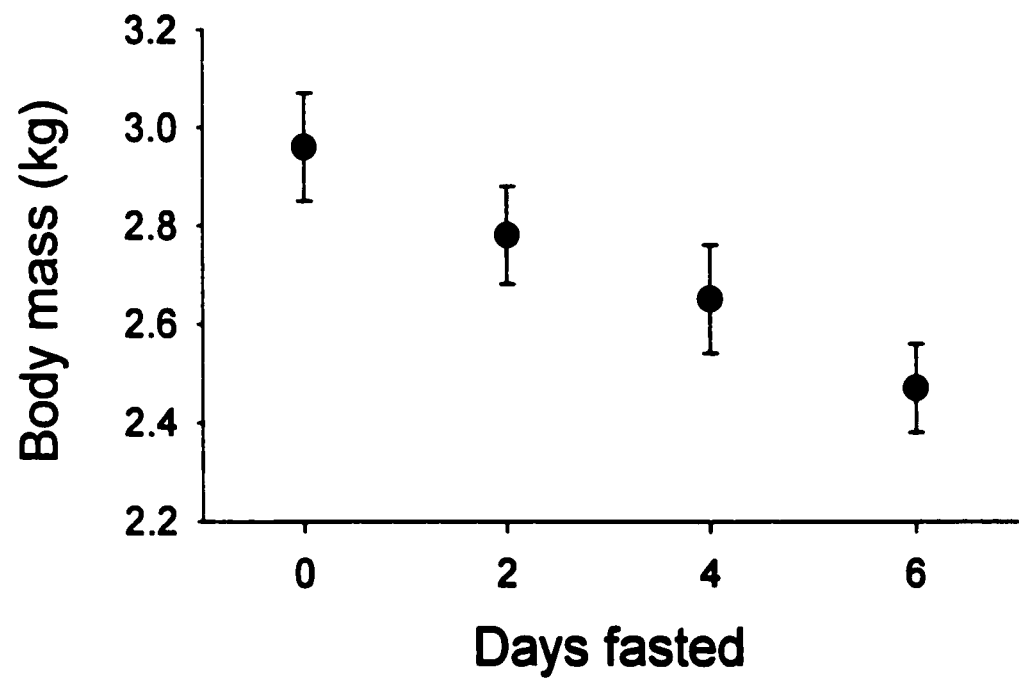


Figure 4.1

**Figure 4.2. Twenty-four hour total oxygen consumption ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) of rabbits**

**before (time=0) and during a six day fast. N=6 for all times except day 6 when n=5.**

**Values are means  $\pm$  SEM and \* indicates a significant difference from the fed value.**

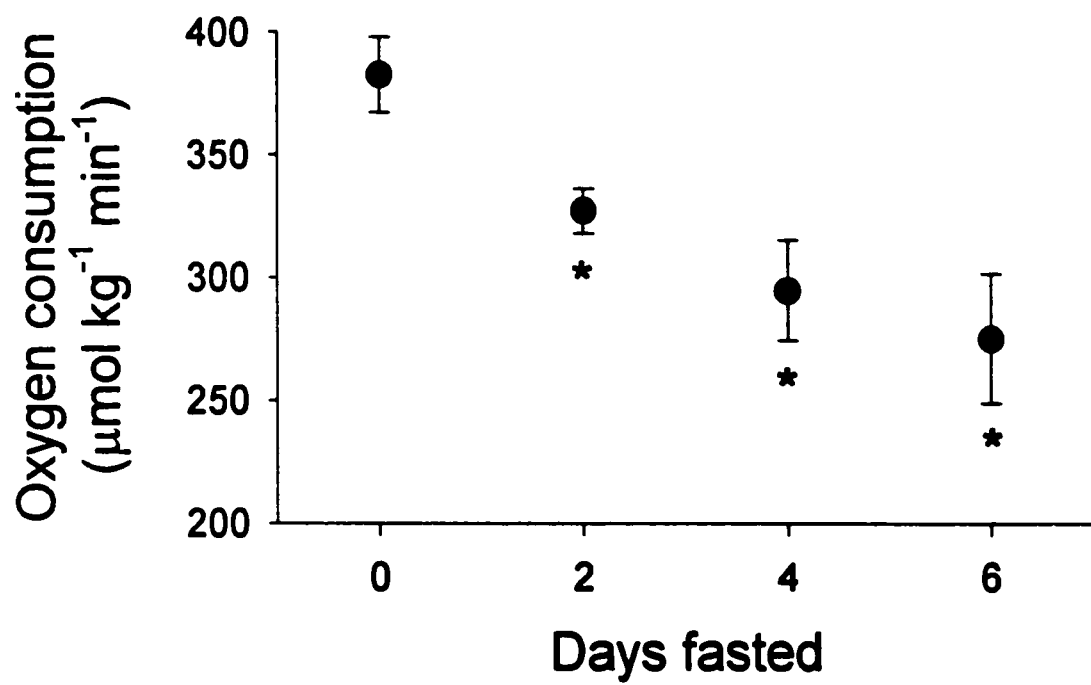


Figure 4.2

**Figure 4.3. Rabbit a) glycerol concentration and b) glycerol rate of appearance or lipolysis before (time=0) and during a six day fast. N=6 for all times except day 6 when n=5. Values are means  $\pm$  SEM and \* indicates a significant difference from the fed value.**

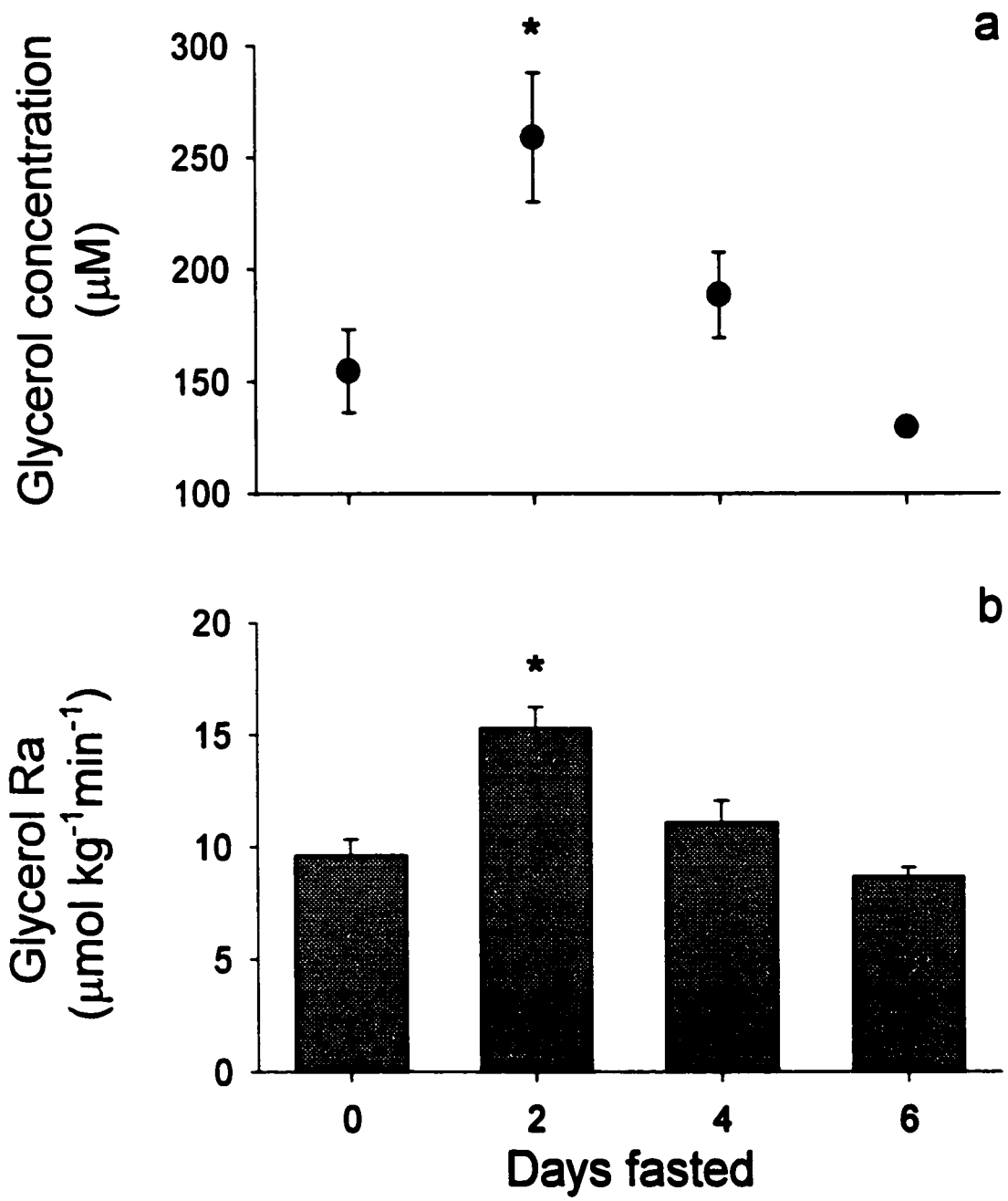


Figure 4.3

**Figure 4.4. Rabbit a) non-esterified fatty acid (NEFA) concentration and b) rate of appearance before (time=0) and during a six day fast. N=6 for all times except day 6 when n=5. Values are means  $\pm$  SEM and \* indicates a significant difference from the fed value.**

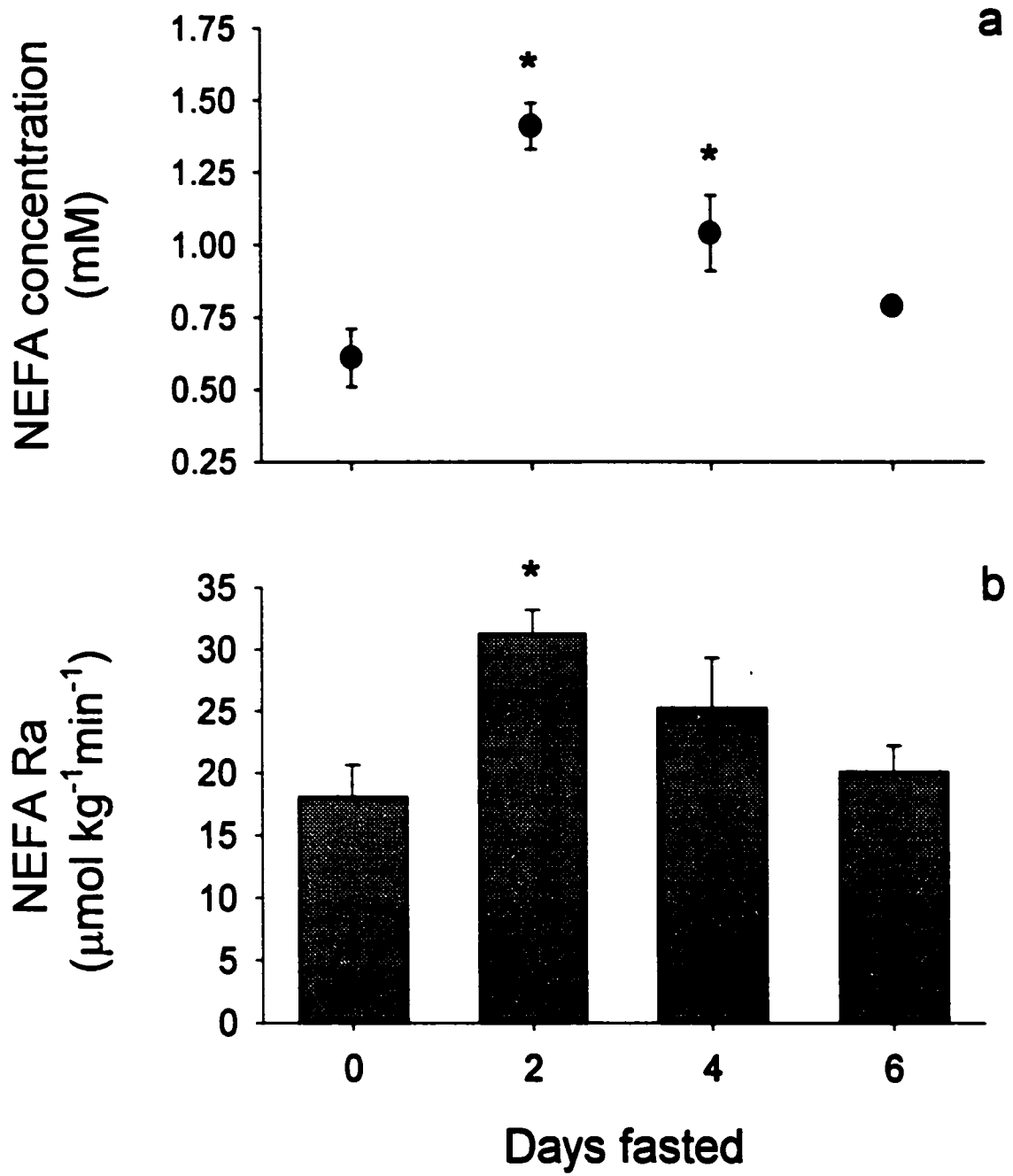


Figure 4.4

**Figure 4.5. Rate of fatty acid oxidation (dark bars) and total fatty acid reesterification (light bars) expressed in a) absolute values ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) and b) relative to the amount of fatty acids made available via triacylglycerol hydrolysis. N=6 for all times except day 6 when n=5. Values are means  $\pm$  SEM and \* indicates a significant difference from the fed value.**

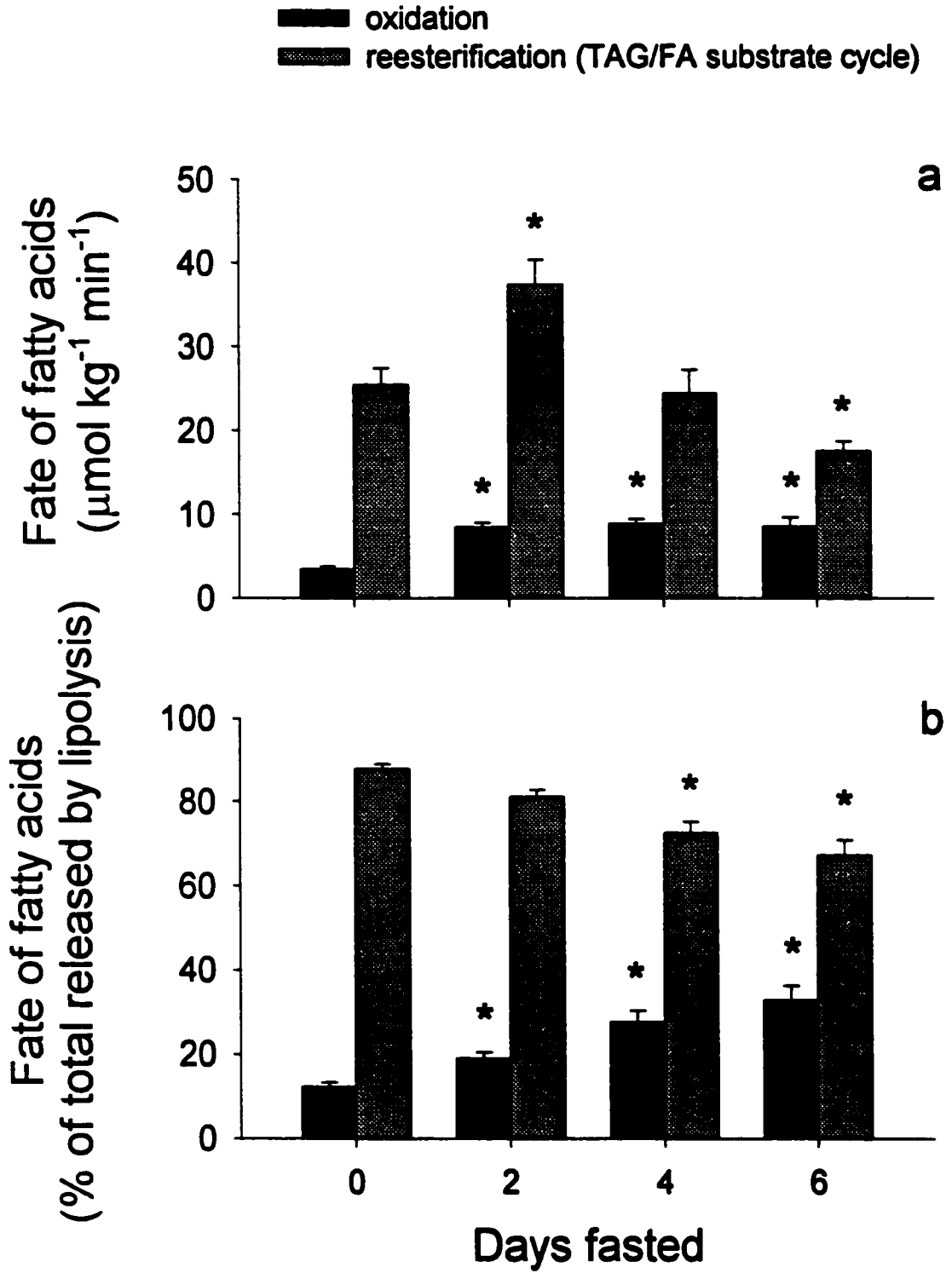


Figure 4.5

**Figure 4.6. Percentage of fatty acids released from triacylglycerol via lipolysis that are reesterified through primary (dark bars) or secondary (light bars) reesterification. N=6 for all times except day 6 when n=5. Values are means  $\pm$  SEM.**

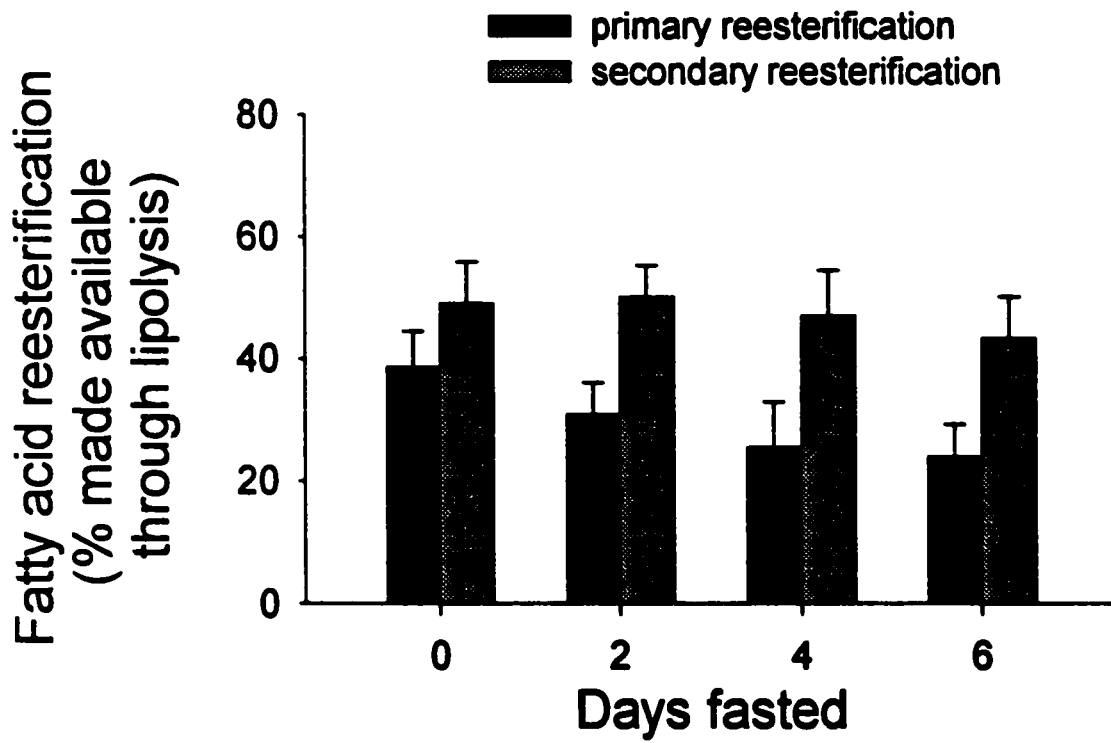


Figure 4.6

**Figure 4.7. The energetic cost of fatty acid reesterification (primary plus secondary) in fed and 6 day fasted rabbits expressed a) in absolute values ( $\text{J kg}^{-1} \text{min}^{-1}$ ) and b) relative to their total energy expenditure.  $N=6$  for all times except day 6 when  $n=5$ . Values are means  $\pm$  SEM and \* indicates a significant difference from the fed value.**

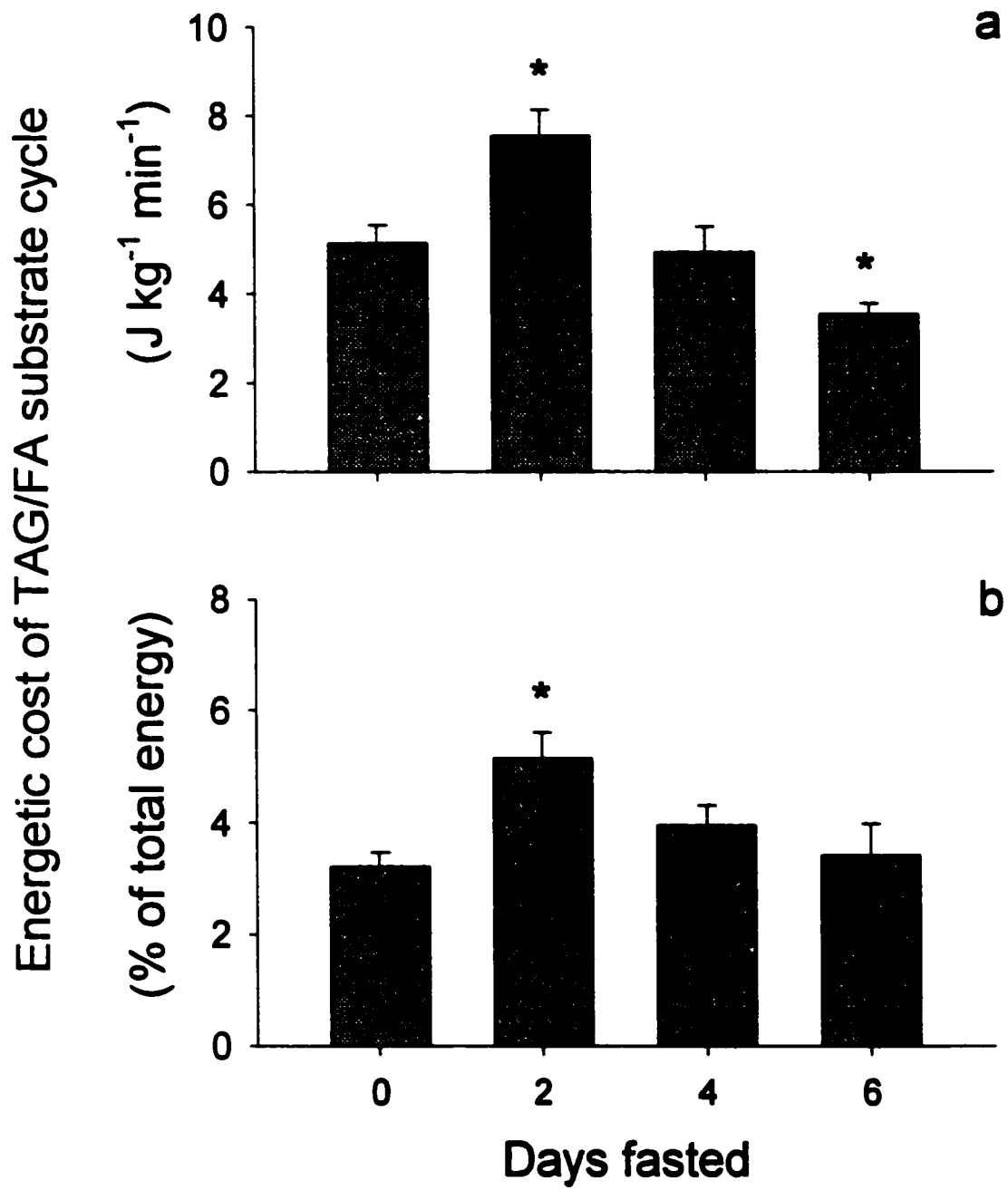


Figure 4.7

## **Discussion**

Many of the studies on fasting mammals that have quantified changes in lipolysis and NEFA rate of appearance have been done on humans where weight loss was less than 5%. In those studies, the rate of lipolysis (Ra glycerol) and fatty acid flux (Ra NEFA) were approximately 2-2.5 times higher in fasting than post-absorptive subjects (Elia *et al.*, 1987; Klein *et al.*, 1990; 1993; 1986). In this study, lipolysis and NEFA flux also increased at the beginning of the fast, however, as the fast progressed these rates decreased back down to the levels that were observed before the fast was started. Decreases in rat plasma glycerol and fatty acid concentrations with long-term fasting suggested that these changes might take place (Parrilla, 1978). Occurring simultaneously with these decreases in triacylglycerol hydrolysis and fatty acid mobilization was a large decrease in energy expenditure. Therefore, it is likely that the changes in rabbit fatty acid mobilization occurred because of a drop in their energy requirements with fasting. Triacylglycerol hydrolysis and fatty acid mobilization decreased all the way back down to rates that were measured in post-absorptive rabbits, yet the animals still had an excess of fatty acids available to them for energy. At the end of six days of fasting, they were only oxidizing about 32% of the fatty acids made usable by lipolysis, with the remainder being reesterified, yet those fatty acids were providing the rabbits with about 85% of their total expended energy.

It is likely that any animal that is able to reduce energy expenditure while fasting will also reduce their rate of fuel mobilization, specifically triacylglycerol hydrolysis and fatty acid mobilization, even though there might be a transitory increase in these rates at the beginning of the fast. It is very common for animals to go through a metabolic

depression when they are fasting to conserve energy, even species that have not evolved in an environment that would encourage adaptation to regular periods of food restriction (Keys *et al.*, 1950; Ma and Foster, 1986; Merkt and Taylor, 1994).

The post-absorptive reesterification rate of available fatty acids in the rabbits in this study was 88% (38% primary and 50% secondary). Wolfe *et al.* (1990) measured slightly lower rates of fatty acid reesterification in humans. They found that about 70% of fatty acids released by lipolysis were reesterified (20% primary and 50% secondary) when subjects were fasted overnight. Two other studies have looked at changes in fatty acid reesterification with fasting. Wolfe *et al.* (1987b) observed an increase in fatty acid primary reesterification rates of humans after having been fasted for 3.5 days. Similarly, Elia *et al.* (1987) observed an increase in the rate of total fatty acid reesterification in humans after four days of fasting. The rabbits in this study also increased their TAG/FA cycling rate on day 2 of the fast. In terms of weight loss, the short periods of food deprivation in the human studies correspond to about two days of fasting in rabbits, therefore, the increase they observed in humans corresponds to the short-term increase that occurred in the rabbits. Wolfe *et al.* (1987b) hypothesized that the rate of TAG/FA substrate cycling may increase with fasting as a mechanism to increase plasma glycerol concentration for gluconeogenesis without flooding the circulation with fatty acids. Unfortunately, it was impossible for me to estimate the contribution of glycerol to gluconeogenesis in the rabbits without direct measurement of the rate of appearance of glucose. This study also shows that temporal changes in lipolysis and fatty acid mobilization that occur with long-term fasting holds true for fatty acid reesterification as well. From its peak on the second day of the fast until the end of the fasting period, the

absolute rate of TAG/FA cycling dropped by more than 50%, and overall, the percent of fatty acids made available by lipolysis that were reesterified dropped from 88% before the fast to only 67% by the end (almost exclusively due to a change in primary reesterification).

Large differences in metabolic rate exist between species of different sizes, therefore, when comparing energy costs it is necessary to use relative values. Before the fast was initiated, rabbit fatty acid reesterification accounted for about 3% of their total energy expenditure. Measurement of non-fasting TAG/FA cycling by others has this substrate cycle accounting for between 0.4 and 3% of total resting energy expenditure in humans and rats (Bahr *et al.*, 1990; Vallerand *et al.*, 1999; Wolfe and Peters, 1987; Wolfe *et al.*, 1987a; 1990; McClelland *et al.*, 2000). After a couple of days of fasting, the total thermogenic contribution of the TAG/FA cycle in rabbits increased to 5%. Similar to rabbits, the energetic cost of the TAG/FA cycle in short-term fasted woodchucks was 4.5% (Chapter 3). Elia *et al.* (1987) found that during a short-term fast the contribution of the TAG/FA substrate cycle to the total energy expenditure of humans increased by more than 6 times. However, in that study there was such a large rise in the rate of cycle with fasting mainly because before the fast was initiated the cost of fatty acid reesterification relative to metabolic rate was extremely low (< 0.4% of total energy expenditure).

The increase at the beginning of a fast of the energetic cost of the TAG/FA substrate cycle relative to energy expenditure is due not only to the increase in fatty acid reesterification, but also the simultaneous decrease in total energy expenditure that usually occurs as soon as a fast is initiated. The TAG/FA substrate cycle is a futile cycle that

consumes energy, therefore, the large decline in the absolute rate of fatty acid reesterification that was measured in the rabbits during the rest of the fast after the initial increase may have been a way to help lower energy expenditure. By itself, the drop in TAG/FA cycling observed here accounted for just over 3% of the difference that was observed in rabbit metabolic rate before and after six days of fasting (differences in TAG/FA cycling and oxygen consumption were  $2.6 \mu\text{mol TAG kg}^{-1} \text{min}^{-1}$  ( $1.6 \text{ J kg}^{-1} \text{min}^{-1}$ ) and  $110 \mu\text{mol O}_2 \text{ kg}^{-1} \text{min}^{-1}$  ( $49 \text{ J kg}^{-1} \text{min}^{-1}$ ), respectively, therefore, the increase in substrate cycling was responsible for  $1.6/49$  or 3.3% of the difference in metabolic rate). This is just one of several substrate cycles that exist in our bodies, it is therefore conceivable that depression of other cycles may also contribute to the metabolic depression that is observed with fasting. It would also be interesting to look at how this and other substrate cycles change during other states of metabolic depression (*e.g.* torpor or hibernation). The role of substrate cycling as a physiological mechanism to increase energy expenditure has been demonstrated in many organisms (Newsholme, 1978; Newsholme and Parry-Billings, 1992), including healthy humans (Bahr *et al.*, 1990; Romijn *et al.*, 1993; Vallerand *et al.*, 1999; Wolfe *et al.*, 1990), burn patients (Wolfe *et al.*, 1987a), malignant-hyperthermic pigs (Clark *et al.*, 1973b) and flying insects (Clark *et al.*, 1973a). This study demonstrates that modification of substrate cycling rates also contributes to lower energy expenditure.

## **CHAPTER 5**

# **REGULATION OF *IN VIVO* LIPID METABOLISM IN RABBITS BY LEPTIN**

## **Introduction**

Numerous hormones regulate lipid metabolism and leptin is the most recently discovered among them (Zhang *et al.*, 1994). This novel hormone is a protein that is primarily produced by adipose tissue and the rate of leptin secretion and its plasma concentration are correlated with total fat mass (Hamilton *et al.*, 1995; Klein *et al.*, 1996; Maffei *et al.*, 1995). Therefore, this hormone circulates as an internal signal indicating the size of body fat stores.

Much research has focused on the fundamental role of leptin as a 'lipostat' in the regulation of body mass. This role in energy balance was discovered when the daily administration of recombinant leptin was found to cause significant weight loss and reduced food intake in *ob/ob* and lean wild-type mice, whereas no such changes were observed in *db/db* mice (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pellymouther *et al.*, 1995). In *ob/ob* mice, the gene coding for leptin is mutated and produces a form of the hormone that is prematurely terminated and, therefore, cannot be recognized by the receptors (Zhang *et al.*, 1994). Obese *db/db* mice have a point mutation in the gene that specifically transcribes for the hypothalamic long isoform of the leptin receptor resulting in suppressed expression of this isoform and increased expression of a much shorter isoform that is unable to activate the proteins responsible for signal transduction and activation of transcription (STAT proteins) (Ghilardi *et al.*, 1996; Lee *et al.*, 1996; Tartaglia *et al.*, 1995). The leptin-induced decrease in food consumption is now known to be mediated primarily through the hypothalamus (Ahima *et al.*, 1996; Stephens *et al.*, 1995). However, *ob/ob* mice chronically injected with leptin showed more than twice the weight

loss and 30% more inguinal fat loss than pair-fed vehicle-treated controls that had consumed the equivalent amount of food (Levin *et al.*, 1996). While body mass changes of leptin-treated lean mice did not differ from pair-fed mice, they did, however, lose 60% more inguinal fat mass than pair-fed controls (Levin *et al.*, 1996). Therefore, leptin regulates overall energy homeostasis in other ways than simply through changes in food intake.

Leptin appears to have an important impact on the relative contribution of the different oxidative fuels available to total energy expenditure. The respiratory quotient of *ob/ob* mice decreases with leptin treatment in a dose-dependent manner, indicating a switch from carbohydrate to lipid oxidation (Hwa *et al.*, 1997). This effect on fuel selection has only been shown in an animal lacking endogenous leptin, although it is likely that leptin would likely have a similar effect in wild-type animals. Subsequently, one of the goals of this study was to measure the effects of leptin on *in vivo* rates of lipolysis, fatty acid mobilization and lipid oxidation.

The higher rates of adipose tissue loss observed in leptin-treated animals can also be partly attributed to increases in metabolic rate. However, only when animals are in certain physiological states does leptin treatment actually cause measurable increases in total energy expenditure. For example, *in vivo* leptin treatment does not cause an increase in total metabolic rate in animals that are fed and awake during the active part of their circadian cycle, yet leptin administration does prevent the lowering of metabolic rate observed during sleep, torpor and fasting (Doering *et al.*, 1998; Geiser *et al.*, 1998; Halaas *et al.*, 1995; Hwa *et al.*, 1997; Mistry *et al.*, 1997; Stehling *et al.*, 1996). Therefore,

leptin-induced increases in metabolic rate do occur, but they are small in relation to total energy expenditure and only seem to happen when metabolic rate would normally be low.

Leptin-induced changes in energy expenditure have primarily been attributed to the action of leptin on the hypothalamic-pituitary-thyroid axis causing an increase in thyroid production of triiodothyronine ( $T_3$ ) (Legradi *et al.*, 1997). This hormone is one of the key regulators of standard metabolic rate (Harper and Brand, 1994; Rothwell *et al.*, 1982), whose effects on energy expenditure are at least partially mediated by changing the proton leakiness of inner mitochondrial membranes (Harper and Brand, 1994). Leptin has also been shown through both direct and indirect action to increase the mRNA expression of numerous uncoupling proteins (UCP's) (Gong *et al.*, 1997; Sarmiento *et al.*, 1997; Scarpace *et al.*, 1997; Zhou *et al.*, 1997). Of these, UCP1 has clearly been shown to increase the thermogenic capacity of brown adipose tissue, again by increasing mitochondrial uncoupling (see review by Himms-Hagen, 1989). In fact, mitochondrial proton leakiness has been estimated from *in vitro* measurements to account for between 15-30% of standard metabolic rate (Rolfe *et al.*, 1994; 1999; Rolfe and Brand, 1996).

Another mechanism by which leptin may increase resting metabolic rates above basal levels include the stimulation of substrate cycles. A substrate cycle occurs when concurrent flux through opposing reactions catalyzed by different non-equilibrium enzymes uses energy without net conversion of substrate to product. Increased substrate cycling has been previously shown to be a source of increased metabolic rate (Clark *et al.*, 1973b; Newsholme and Crabtree, 1976; Wolfe *et al.*, 1987a). TAG hydrolysis (lipolysis) releases fatty acids to be used for either oxidative metabolism or reesterification (TAG resynthesis). Simultaneous lipolysis and reesterification form the TAG/FA cycle, a

substrate cycle which can dissipate energy for thermogenesis or weight regulation (Newsholme and Crabtree, 1976; Wolfe *et al.*, 1987a). Leptin could either decrease reesterification to channel more fatty acids towards oxidation, a strategy supported by *in vitro* measurements of fatty acid oxidation (Bai *et al.*, 1996; Muoio *et al.*, 1997; Sarmiento *et al.*, 1997; Shimabukuro *et al.*, 1997), or it could stimulate reesterification to increase TAG/FA cycling and energy expenditure. Although, the direct effects of leptin on substrate cycling rates have not yet been investigated, it has been shown that the triacylglyceride/free fatty acid (TAG/FFA) substrate cycling rate of human adipocytes is negatively correlated with obesity (Bottcher and Furst, 1997). *In vitro* leptin treatment also increases the ratio of glycerol:fatty acids that are released from isolated adipocytes (Wang *et al.*, 1999). These results suggest that the TAG/FFA cycling rate may be increased by leptin, therefore, the primary goal of this study was to measure the effects of leptin on the cycling rate of this substrate cycle.

## Methods

### *Animals and surgery*

All the rabbits used in this study were New Zealand white males. Food and water intake was recorded for each rabbit for 3 days prior to surgery and each day following. They were fed rabbit chow (52% carbohydrate, 3% fat, 16% protein, 14% crude fiber, 15% moisture). On the morning of surgery, the rabbits were pre-medicated with an intramuscular injection of ketamine (15 mg kg<sup>-1</sup>), midazolam (0.5 mg kg<sup>-1</sup>) and robinel (0.005 mg kg<sup>-1</sup>), placed under halothane/isoflurane anesthesia with oxygen and intubated. Using sterile surgical technique I isolated the right jugular vein and right carotid artery and fed

into each vessel saline filled PE-50 cannula approximately 7 cm towards the heart. Both cannulas were sutured to the vessels, tunneled under the skin, exteriorized through a small hole between the shoulder blades and sutured in place. The cannulas were filled with sterile saline containing heparin ( $40 \text{ units ml}^{-1}$ ) and penicillin G ( $125\,000 \text{ units ml}^{-1}$ ), coiled and secured to the animal between its shoulder blades. The rabbits were given buprenorphine ( $0.02 \text{ mg kg}^{-1}$  subcutaneous) on the morning and afternoon of the day of surgery and 1 day following to eliminate post-operative pain. To keep the cannulas patent, the heparinized saline was withdrawn each day, flushed with saline and re-filled with heparinized saline. Because heparin has lipolytic properties the heparinized saline was always removed from the cannulas before any solution was flushed into the animal. The rabbits were used for an experiment 3-5 days following surgery and always after food and water consumption had been at pre-operative levels for at least 2 days.

### *Indirect calorimetry*

A fed rabbit was placed in a closed Plexiglas respirometer (54 x 38 x 67 cm), the cannulas were uncoiled and made accessible from outside the respirometer, and the lid was closed. The respirometer was supplied with a known amount of room air that was circulated inside the chamber with a small enclosed fan in the lid, and the chamber was connected to an indirect calorimetry system (Columbus Instruments; Columbus, Ohio, USA). The oxygen and carbon dioxide sensors were calibrated using gases of known concentrations and measurement of whole animal oxygen consumption and carbon dioxide production was started (described previously by Fournier and Weber (1994)). Every 5 min until the end of the experiment, a 90 sec average of  $\text{O}_2$  consumption and  $\text{CO}_2$

production was recorded on a computer. All urine was collected as it passed through the metal floor of the respirometer and funneled into an attached polyethylene bottle kept on ice.

Thirty minutes after the rabbit was placed in the respirometer, an injection of rat recombinant leptin (R&D Systems, Minneapolis, MN, USA) ( $1 \text{ mg kg}^{-1}$  mixed in 5 ml saline;  $n=6$ ) or saline (5 ml;  $n=6$ ) into the venous cannula was given over a period of 10 min. This dose of leptin has been previously used in rodents with no adverse side effects (Frühbeck *et al.*, 1998). Following the injection, the respirometer was briefly opened and the cannulas were coiled and reattached to the back of the rabbit. The rabbit was then left alone in the closed respirometer for the 12 hours. No food was provided, though water was freely available.

#### *Continuous infusion*

12 hours later the respirometer was briefly opened, the cannulas were again uncoiled and made accessible from outside the respirometer, and the lid was closed. The venous cannula was connected to a 10 cc syringe on a calibrated syringe pump (Harvard Apparatus; South Natick, MA, USA) set at  $5 \text{ ml h}^{-1}$ . The syringe contained an infusate of trace amounts of  $2\text{-}^3\text{H}$  glycerol ( $8.06 \pm 0.34 \text{ } \mu\text{Ci ml}^{-1}$ ) and  $1\text{-}^{14}\text{C}$  palmitate ( $8.96 \pm 0.33 \text{ } \mu\text{Ci ml}^{-1}$ ). The infusate was prepared daily by mixing  $1\text{-}^{14}\text{C}$  palmitate with rabbit albumin dissolved in saline (0.9% NaCl), adding  $2\text{-}^3\text{H}$  glycerol and making the solution up to 5 ml with saline. The radioactivity of each infusate was determined by counting it on a Tri-Carb 2500 B-counter (Packard, Canada). Forty minutes after the start of the infusion the arterial cannula was used to take a 1.5 ml blood sample every 5 min for 15 min.

Immediately after sampling, the blood was centrifuged and the plasma was separated and stored in glass containers (to prevent glycerol absorption) at -20°C for analysis a few days later. Measurement of indirect calorimetry was continued throughout the infusion.

### *Analyses*

Plasma palmitate, total non-esterified free fatty acid (NEFA) and glycerol concentration and radioactivity were measured as described previously in Chapter 3. Steady-state conditions existed during blood sampling as changes in metabolite concentration, specific activity or rate of appearance were not observed over time. Therefore, measurements for each individual were averaged following analysis.

Rabbit leptin immunoassay kits are not available, therefore, leptin concentration was measured using an immunoassay kit (Quantikine M; R&D Systems, Minneapolis, MN, USA) employing the sandwich enzyme immunoassay technique and a polyclonal antibody specific for mouse (*Mus musculus*) leptin. Of the 167 total, amino acids 49 ⇒ 138 of rabbit leptin have been sequenced (Christian Doyan, personal communication; University of Ottawa, Canada), and are part of the 146 amino acid peptide that circulates in the blood (21 amino acids are cleaved when leptin is secreted). Of these known amino acids for rabbit leptin, mouse is 79% identical. Therefore, because the antibody in the immunoassay is not specific for rabbit leptin, it is possible that the measured values of rabbit leptin concentration in this study may be underestimated, however, they will still be useful in comparing the magnitude of difference that could exist between treatment groups.

### *Calculations*

Lipid and carbohydrate oxidation rates were calculated according to Frayn (1983).

$$\text{Glucose oxidation (g min}^{-1}\text{)} = 4.55 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 3.21 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 2.87 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

$$\text{Lipid oxidation (g min}^{-1}\text{)} = 1.67 \times \text{VO}_2 \text{ (l min}^{-1}\text{)} - 1.67 \times \text{VCO}_2 \text{ (l min}^{-1}\text{)} - 1.92 \times \text{urinary N excretion (g min}^{-1}\text{)}$$

The second equation assumes that all lipid oxidation is of palmitoyl-stearoyl-oleoyl-glycerol, the average fatty acid found in human adipose tissue. Assuming this is correct, oxidation rates can were then converted to their molar equivalent by using the molecular weight of this triacylglycerol (861 g mol<sup>-1</sup>) and glucose (180 g mol<sup>-1</sup>). Finally, fatty acid oxidation was calculated by multiplying the molar rate of triacylglycerol oxidation by 3 as each mole of triacylglycerol contains 3 moles of fatty acids. Urinary nitrogen excretion was measured by the Kjeldahl method. However, only 4 rabbits urinated during the 12 h experiment so these values were averaged with a rate of 24 h urinary nitrogen excretion that was measured on a separate group of rabbits (n=11). This nitrogen excretion rate was 24 μmol kg<sup>-1</sup> min<sup>-1</sup>.

The rates of appearance (Ra) of glycerol and palmitate were calculated using the steady state equation of Steele (1959).

$Ra$  ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) = isotope infusion rate ( $\text{DPM kg}^{-1} \text{min}^{-1}$ ) / plasma specific activity (SA;  $\text{DPM } \mu\text{mol}^{-1}$ );

SA = plasma isotope activity ( $\text{DPM ml}^{-1}$ ) / plasma metabolite concentration ( $\mu\text{mol ml}^{-1}$ );

DPM= disintegrations per minute

$Ra$  glycerol is assumed to be a direct measure of triacylglycerol hydrolysis as adipose tissue lacks glycerokinase and cannot metabolize glycerol, and incomplete hydrolysis of triacylglycerol into mono- and diacylglycerol is negligible (Arner and Ostman, 1974). This means that all the glycerol released by triacylglycerol hydrolysis should appear in the plasma.  $Ra$  NEFA was determined by dividing  $Ra$  palmitate by the fractional contribution of palmitate to total NEFA. The rate of total NEFA reesterification was calculated as the difference between  $3 \times Ra$  glycerol (total NEFA released from triacylglycerol) and the simultaneous rate of FA oxidation (see below). Steady-state conditions existed during blood sampling as changes in metabolite concentration, specific activity or rate of appearance were not observed over time. The samples for an individual were averaged following analysis. Rates of primary fatty acid reesterification (when fatty acids that are hydrolyzed from TAG are reesterified back into TAG without entering the circulation) and secondary reesterification (fatty acids that undergo lipolysis, enter the circulation and are then reesterified back into TAG in a different cell) were calculated according to Wolfe *et al.* (1990):

$$\text{Primary TAG:FA cycling} = (3 \times Ra \text{ glycerol}) - Ra \text{ NEFA,}$$

**Total cycling = (3 × Ra glycerol) - FA oxidation rate,**

**Secondary TAG:FA cycling = total cycling - primary cycling.**

These calculations also assume that any fatty acids that are released by lipolysis will either be reesterified or move into the circulation. This is important because oxidation of fatty acids by adjacent tissue before movement into the plasma pool would result in an underestimation of Ra NEFA. Fortunately, there is evidence to show that fatty acid oxidation by this route is probably small (Wolfe *et al.*, 1990). Any values of total TAG/FA recycling should be accurate, as it does not depend upon either Ra NEFA or the site of fatty acid oxidation.

I also calculated the energetic cost of fatty acid reesterification knowing that the TAG/FA substrate cycle costs approximately 603 kJ mol<sup>-1</sup> (144 kcal mol<sup>-1</sup>) of recycled triacylglycerol (Wolfe *et al.*, 1990). This is because the TAG/FA cycle uses approximately eight high-energy phosphate bonds per mole of recycled triacylglycerol and about 75 kJ (18 kcal) of heat are released per mole of hydrolyzed ATP. Finally, oxygen consumption (μmol kg<sup>-1</sup> min<sup>-1</sup>) was converted to energy expenditure (J kg<sup>-1</sup> min<sup>-1</sup>; 0.45 J of heat per μmol of oxygen) so the contribution of this substrate cycle to the total energy expenditure of the animal could be calculated.

### *Statistics*

Statistical differences of oxygen consumption were tested for by using a repeated measures ANOVA and confirmed with the Bonferroni post-hoc test. Student's t-tests were used to identify the other statistical differences. All data expressed as percentages

were transformed to the arcsine of their square root before testing for differences (Zar, 1984). Statistical significance is indicated when  $p < 0.05$ . Results are presented as means  $\pm$  SEM.

## Results

Oxygen consumption of the leptin treated rabbits during the twelve hours after the injection did not differ from the oxygen consumption that was measured before the treatment; however, the rabbits that were treated with saline did reduce their metabolic rate over time ( $p < 0.05$ ; Fig. 5.1).

The lipolytic rate (Ra glycerol) of the leptin-treated rabbits was 53% higher than the control rabbits ( $9.66 \pm 0.62$  versus  $14.78 \pm 0.93 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ;  $p < 0.05$ ; Fig. 5.2b) 12 hours after treatment. No change in plasma glycerol concentration occurred with the increased rate of appearance of glycerol from the triacylglycerol stores (Fig. 5.2a). The increase in lipolytic rate with leptin treatment also resulted in a 40% higher rate of appearance of fatty acids into the blood ( $20.69 \pm 2.14$  versus  $29.03 \pm 3.03 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ;  $p < 0.05$ ; Fig. 5.3b). Unlike glycerol, the increase in fatty acid flux did result in an increase in the plasma concentration of these non-esterified fatty acids. Total plasma fatty acid concentration of the rabbits injected with leptin was 66% higher than that of the rabbits injected with saline ( $p < 0.05$ ; Fig. 5.3a). Despite the difference in total NEFA concentration, leptin treatment did not cause a difference in the composition of major plasma fatty acids (Table 5.1).

The increase in basal oxygen consumption was also accompanied with a shift in the contribution of the different oxidative fuels to aerobic metabolism. No difference in

carbohydrate and lipid metabolism existed between the 2 groups of rabbits prior to injection; however, 12 hours later the leptin treated rabbits had a 55% higher rate of fatty acid oxidation ( $7.15 \pm 0.83 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ) than that of the control rabbits ( $4.63 \pm 0.42 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ;  $p < 0.05$ ; Fig. 5.4a). However, because the oxygen consumption of the rabbits treated with leptin was higher than that of the control group it is more appropriate to look at the lipid and glucose oxidation rates of the rabbits relative to their energy expenditure. When fatty acid and glucose oxidation are calculated relative to total energy expenditure, there are still differences between the 2 groups. In the leptin rabbits versus the control rabbits the contribution of lipid oxidation to metabolic rate was 33% higher, while the contribution of glucose oxidation was 28% lower ( $p < 0.05$ ; Fig. 5.4b).

The TAG/FA substrate cycling rate (primary plus secondary) was 50% higher in the leptin-treated rabbits compared to the control rabbits 12 hours following injection ( $p < 0.05$ ; Figure 5.5a). Interestingly, when the cycling rate is calculated relative to the amount of fatty acids made available through lipolysis there is no difference between the two groups (Figure 5.5b). This is because there is a concurrent increase in lipolytic rate with the increase in TAG/FA cycling rate. When the TAG/FA substrate cycle is divided into primary and secondary reesterification, the increase of this substrate cycle can be mainly attributed to an increase in primary reesterification ( $p < 0.05$ ; Fig. 5.6). The leptin-treated group had a rate of primary reesterification 85% higher than the group injected with saline. Secondary reesterification also increased by 31% with treatment, although this was not statistically significant.

In the leptin treated rabbits, the energetic cost of this substrate cycle was 50% higher than in the control rabbits ( $7.49 \pm 0.50$  vs.  $5.01 \pm 0.35$ ;  $p < 0.05$ ; Fig. 5.7a). This

accounted for  $4.26 \pm 0.31\%$  of the total energy expenditure of the leptin treated rabbits, compared to  $3.00 \pm 0.21\%$  for the saline treated rabbits ( $p < 0.05$ ; Fig. 5.7b). Although this is a small difference, it actually accounts for 15% of the difference in energy expenditure that was observed with leptin treatment.

Leptin concentration varies with body fat content, and in humans it has been shown to range anywhere from 1 to 30 ng ml<sup>-1</sup> (Klein *et al.*, 1996). Previous measurements of rabbit leptin concentration have not been made, but in this study the plasma leptin concentration of the leptin treated animals was  $4.84 \pm 1.16$  ng ml<sup>-1</sup> compared to  $0.56 \pm 0.05$  ng ml<sup>-1</sup> in the control animals ( $p < 0.05$ ). Even if the concentrations were not exact due to problems with the assay, I still know that 12 hours following treatment the leptin rabbits had almost 7 times more circulating leptin than the control rabbits.

**Table 5.1. Plasma fatty acid composition (% of total) of rabbits twelve hours following treatment with saline (n=6) or leptin (n=6). Values are means  $\pm$  SEM.**

<b>FATTY ACIDS</b>	<b>CONTROL</b>	<b>LEPTIN</b>
16:0 (palmitate)	29.2 $\pm$ 0.52	29.3 $\pm$ 0.64
18:0 (stearate)	14.8 $\pm$ 0.87	17.5 $\pm$ 1.43
16:1 (palmitoleate)	2.70 $\pm$ 0.33	2.40 $\pm$ 0.48
18:1 (oleate)	22.8 $\pm$ 0.50	20.5 $\pm$ 0.96
18:2 (linoleate)	22.1 $\pm$ 0.53	19.4 $\pm$ 1.21
18:3 (linolenate)	8.50 $\pm$ 0.86	10.8 $\pm$ 0.36

**Figure 5.1. Oxygen consumption of rabbits before (time=0) and following an intravenous injection of either saline (closed circles; n=6) or leptin (open circles; n=6). Each point is the mean value for the previous hour and \* indicates a significant difference from the control value (time=0) for that treatment. Values are means  $\pm$  SEM.**

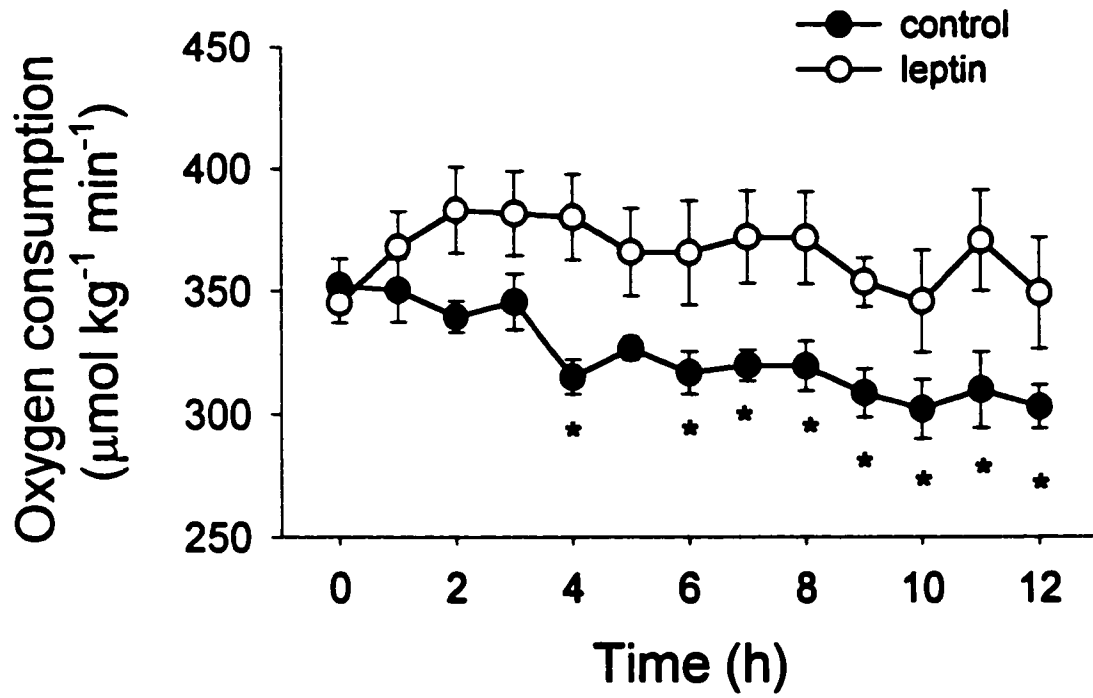


Figure 5.1

**Figure 5.2. a) Glycerol concentration and b) glycerol rate of appearance (lipolysis) in rabbits 12 h following injection of saline (n=6) or leptin (n=6). Values are means  $\pm$  SEM and \* indicates a significant difference between treatments.**

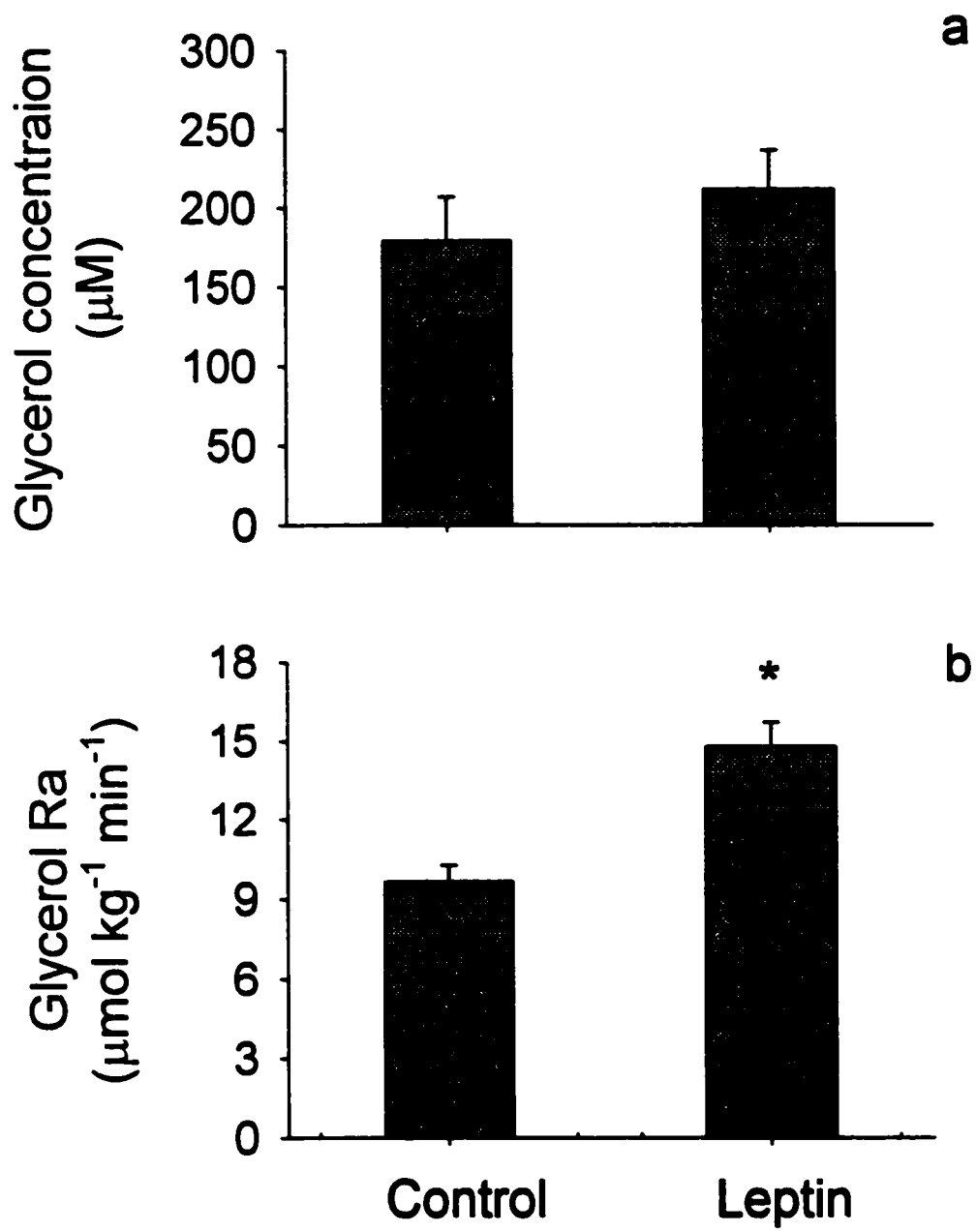


Figure 5.2

**Figure 5.3. a) Non-esterified fatty acid (NEFA) concentration and b) NEFA rate of appearance in rabbits 12 h following injection of saline (n=6) or leptin (n=6). Values are means  $\pm$  SEM and \* indicates a significant difference between treatments.**

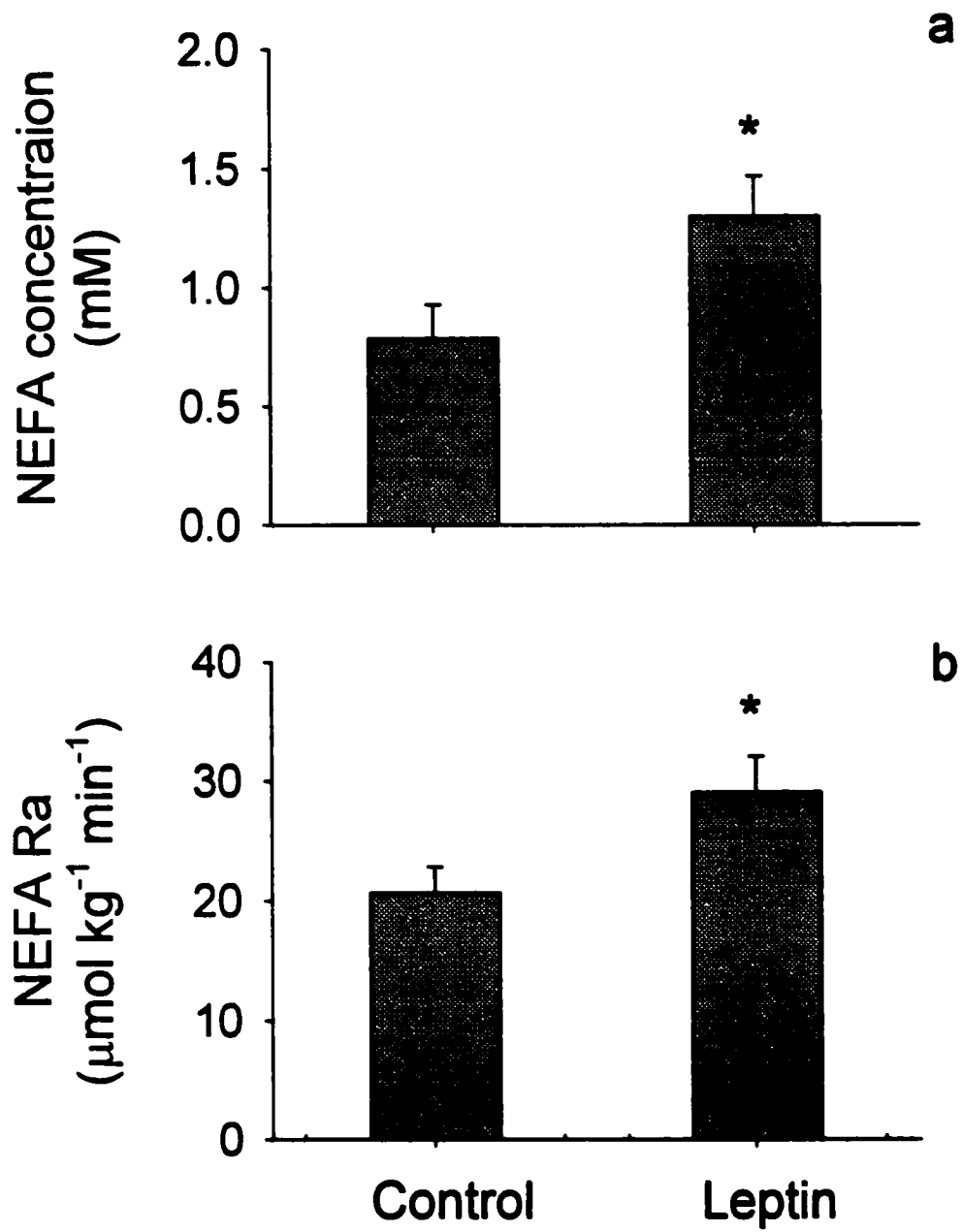


Figure 5.3

**Figure 5.4. Carbohydrate and lipid oxidation of rabbits 12 h following injection of saline (dark bars; n=6) or leptin (light bars; n=6) expressed as a)  $\mu\text{mol}$  (fatty acid or glucose)  $\text{kg}^{-1} \text{min}^{-1}$  and b) their relative contribution to total oxygen consumption. Values are means  $\pm$  SEM and the \* indicates a significant increase in fatty acid oxidation of the leptin treated group.**

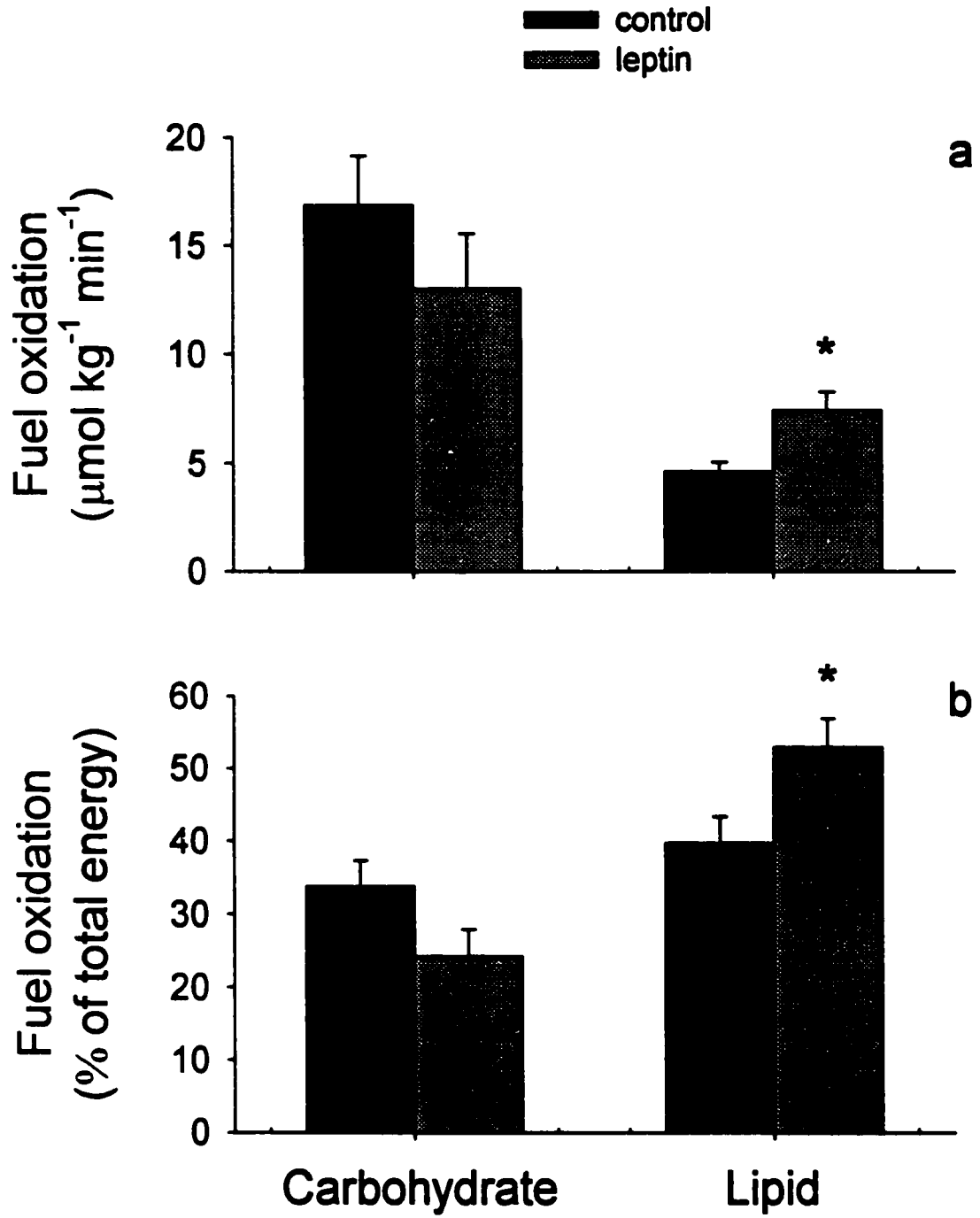


Figure 5.4

**Figure 5.5. Rate of fatty acid oxidation (dark bars; n=6) and total fatty acid reesterification (light bars; n=6) 12 h following injection of saline or leptin expressed in a) absolute values ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) and b) relative to the amount of fatty acids made available through triacylglycerol hydrolysis. Values are means  $\pm$  SEM and \* indicates a significant difference between treatments.**

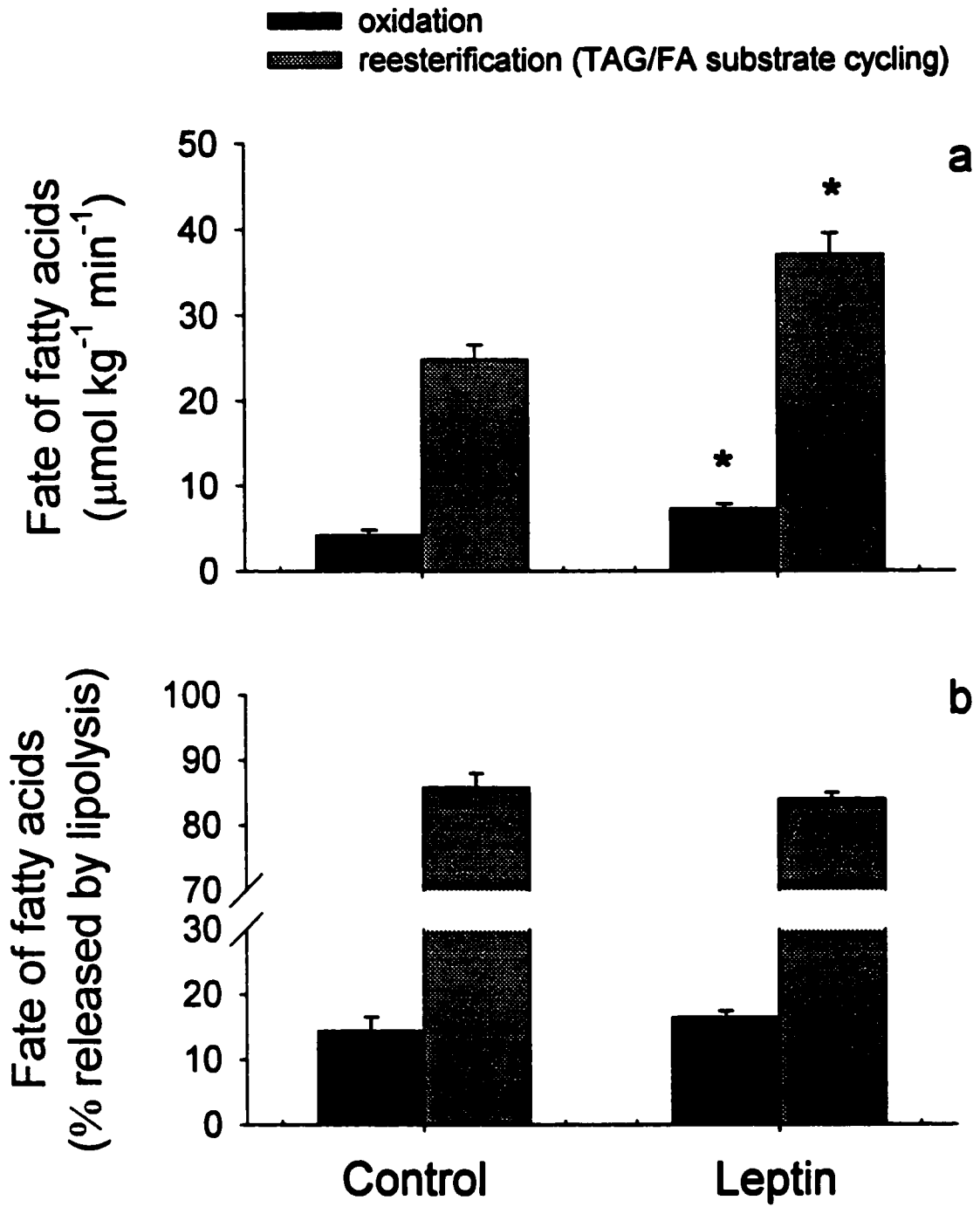


Figure 5.5

**Figure 5.6. Absolute values of primary (dark bars; n=6) and secondary (light bars; n=6) fatty acid reesterification in rabbits 12 h following injection of saline or leptin. Values are means  $\pm$  SEM and \* indicates a significant increase in primary reesterification with leptin treatment.**

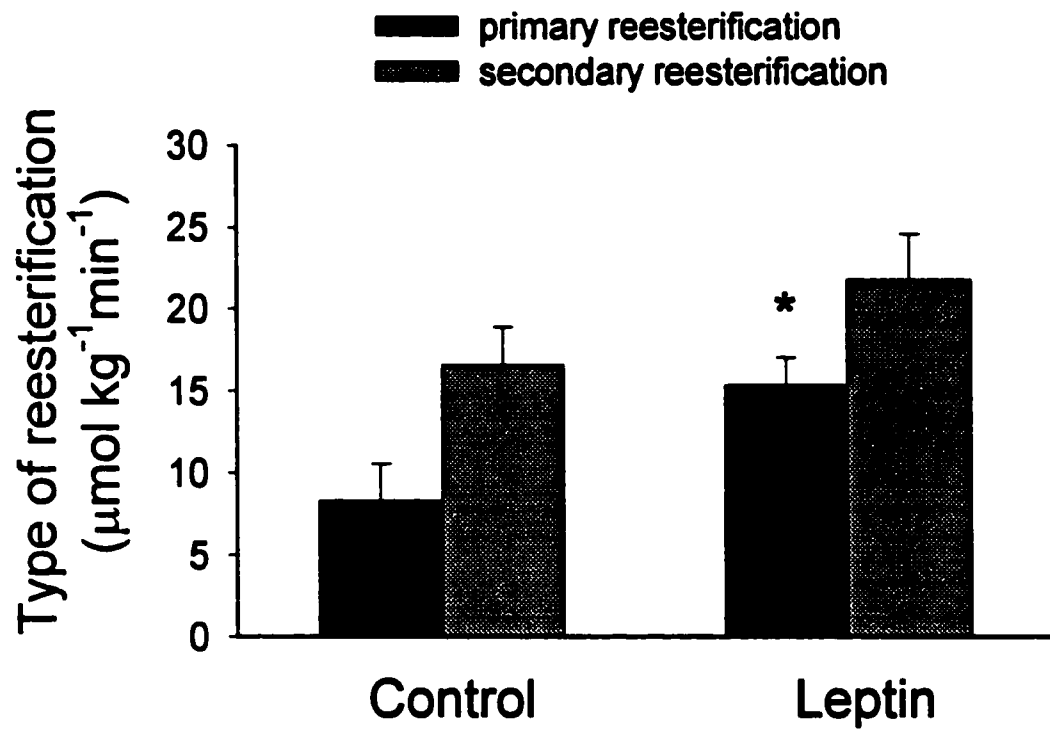


Figure 5.6

**Figure 5.7. a) The energetic cost of the TAG/FA substrate cycle expressed in absolute values ( $\text{J kg}^{-1} \text{min}^{-1}$ ) and b) the energetic cost of the TAG/FA substrate cycle expressed relative to the total energy expenditure of the rabbits 12 h following injection of saline (n=6) or leptin (n=6). Values are means  $\pm$  SEM and \* indicates a significant difference between treatments.**

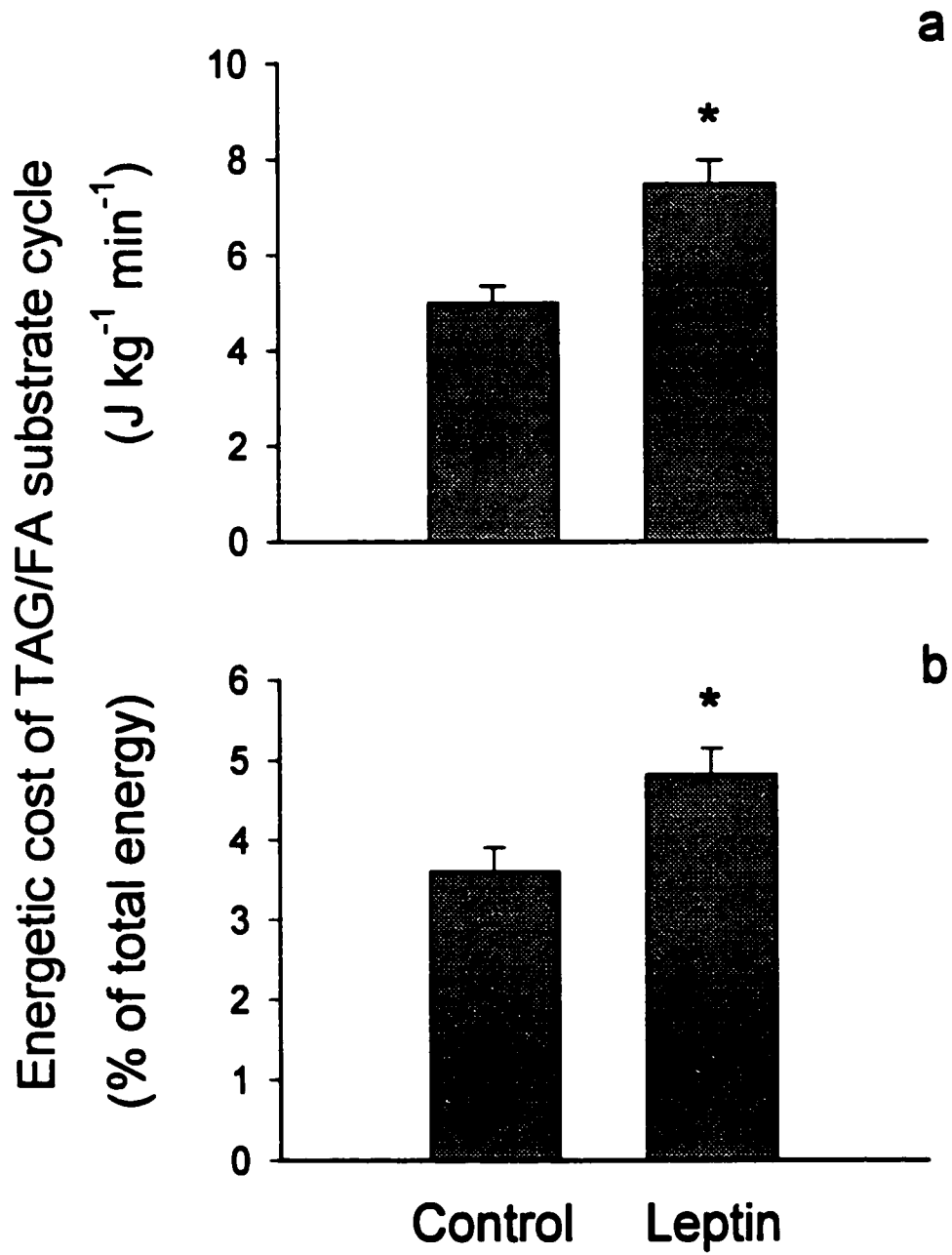


Figure 5.7

## **Discussion**

Leptin treatment has been shown to prevent energy expenditure from dropping during physiological states when metabolism would normally be low (*e.g.* following absorption of a meal or during sleep) and does not appear to increase it above active rates (Doering *et al.*, 1998; Geiser *et al.*, 1998; Halaas *et al.*, 1995; Hwa *et al.*, 1997; Mistry *et al.*, 1997; Stehling *et al.*, 1996). I also observed this effect, as no change in oxygen consumption was measured in the leptin-treated rabbits at the end of the experiment compared to the beginning, whereas the rabbits treated with saline did have a significant decrease in oxygen consumption by the end of the experiment. The decline in energy expenditure is a normal post-absorptive fasting response that is blocked by leptin. Although triiodothyronine (T<sub>3</sub>) concentrations were not measured in this study, leptin has previously been shown to increase thyroid production of this hormone via the hypothalamus (Legradi *et al.*, 1997). This hormone is a key regulator of standard metabolic rate (Harper and Brand, 1994; Rothwell *et al.*, 1982) and may have been one of the mechanisms through which leptin was able to alter the energy expenditure of the treated rabbits in this study.

Leptin is also thought to be able to change the proton leakiness of membranes and, hence, energy expenditure by varying the mRNA expression and membrane concentration of uncoupling protein (UCP). Different uncoupling proteins are expressed in specific tissues and are affected by leptin through different pathways. UCP1 is only expressed in brown adipose tissue and, thus far, is the only UCP that has been clearly shown to increase the thermogenic capacity of animals (see review by Himms-Hagen, 1989). Leptin administration causes an increase in UCP1 mRNA levels in brown adipose tissue and

augments energy expenditure (Scarpace *et al.*, 1997). It was demonstrated that the leptin-induced increase in UCP1 is mediated through the sympathetic nervous system because leptin is unable to increase uncoupling protein mRNA in cultured brown adipocytes and denervated brown adipose tissue (Collins *et al.*, 1996; Scarpace and Matheny, 1998; Shimuzu *et al.*, 1997).

The expression of uncoupling proteins other than UCP1 is also affected by leptin. Prolonged hyperleptinemia in wild-type mice causes an increase in white adipose tissue mRNA expression of UCP2 (Sarmiento *et al.*, 1997; Zhou *et al.*, 1997), an uncoupling protein expressed in most tissues. Similarly, leptin treatment increases muscle and brown adipose tissue UCP3 mRNA (Gong *et al.*, 1997). This uncoupling protein is found almost exclusively in these two tissues. While UCP1 is indirectly regulated by leptin via the sympathetic nervous system, T<sub>3</sub> is probably the main controller of UCP3 expression. UCP3 mRNA levels are correlated with plasma T<sub>3</sub> levels (Gong *et al.*, 1997) and T<sub>3</sub> production is regulated by leptin via the hypothalamic-pituitary-thyroid axis (Legradi *et al.*, 1997). There is also evidence that fatty acids upregulate UCP2 and UCP3 gene expression in certain muscle types (Samec *et al.*, 1998; Weigle *et al.*, 1998), maybe as a supply and demand response to a high level of available substrate.

Fatty acids have also been shown to be stimulators of cellular respiration (Nobes *et al.*, 1990), therefore, leptin may also indirectly increase metabolic rate through its lipolytic effects. Surprisingly, the rate of fatty acid oxidation observed in the leptin treated rabbits was almost as high as that observed in rabbits that had been fasting for an entire week ( $7.15 \pm 0.83$  vs.  $8.54 \pm 1.06 \mu\text{mol FA kg}^{-1} \text{min}^{-1}$ ; this study vs. Chapter 4). A switch from carbohydrate to lipid oxidation with leptin treatment has been previously shown in an

animal lacking endogenous leptin (Hwa *et al.*, 1997). In this study, the shift in primary fuel use from glucose to lipids occurred with a leptin-induced increase in lipolytic rate. This increase in lipolysis occurred with no concurrent increase in plasma glycerol concentration. This indicates that glycerol uptake must have also increased in these animals, probably in the liver as hepatocytes have the capacity to use glycerol. Such a change was not observed with plasma NEFA concentration. Interestingly, while increases in plasma fatty acid occur with leptin treatment in rabbits (this study) and sheep (Henry *et al.*, 1999), they do not appear in rats (Chen *et al.*, 1996; Shimabukuro *et al.*, 1997). Nevertheless, the *in vivo* changes in lipolytic rate and fatty acid flux provide substrate for the increased oxidative demand for lipids brought on by 1) the increase in energy expenditure and 2) the switch in fuel use.

The *in vivo* increases in lipolysis and fatty acid mobilization that I measured in this study have previously only been measured on *in vitro* cell preparations. Frühbeck *et al.* (1997) was the first to show an auto or paracrine mode of action by leptin when they measured changes in the lipolytic rates of cultured adipose tissue with leptin treatment. When lean mouse adipocytes were treated with leptin, lipolytic rate increased by 28%, whereas *ob/ob* mouse lipolytic rate increased 123%. As expected, no change occurred in the *db/db* mouse adipocytes that lacked a functional leptin receptor. They demonstrated that the lipolytic effect of leptin on adipocytes is not dependent upon hypothalamic, neural or adrenergic control, although these components certainly modify *in vivo* leptin effects. A similar study was done by Siegrist-Kaiser *et al.* (1997) who measured the lipolytic rates of *ex vivo* adipocytes of wild-type and *fal/fa* rats with or without leptin treatment (*fal/fa* rats do not possess a functional leptin receptor). As with the mice, the adipocytes of the

wild-type rats increased their lipolytic rate following exposure to leptin, whereas no change was observed in the adipocytes lacking a functional receptor. In another study, Frühbeck *et al.* (1998) injected lean, *ob/ob* and *db/db* mice with three different doses of leptin before isolating adipocytes and measuring lipolytic rates. Again, no effect of leptin treatment was observed in the *db/db* mice, the two highest doses induced a change in the *ob/ob* mice and only the highest dose caused a significant increase in lipolysis in the wild-type mice. The fact that the lipolytic rates of the *ob/ob* adipocytes were much more strongly affected by leptin than the wild-type adipocytes in both their experiments, suggests that, in the absence of a functional leptin protein as is the case in *ob/ob* mice, the receptor is strongly upregulated.

Leptin appears to be able to mediate these changes in fatty acid metabolism by changing enzyme mRNA levels and concentration. For example, the presence of leptin inhibits the expression of acetyl-CoA carboxylase in adipocytes (Bai *et al.*, 1996). This is the rate-limiting enzyme for long chain fatty acid synthesis and it is essential for the conversion of carbohydrate to fatty acids and caloric storage as triacylglycerol. Under basal conditions (no exogenous hormones added to the medium), cultured differentiating adipocytes expressing the *ob* gene have lower acetyl-CoA carboxylase, and fatty acid and triacylglycerol synthesis compared to cells that do not express the leptin gene. Using a different experimental approach, long-term treatment of wild-type mice with high concentrations of leptin increased mRNA expression of hormone-sensitive lipase, the key lipolytic enzyme, while causing a decrease in mRNA expression of the lipogenic enzyme, fatty acid synthase (Sarmiento *et al.*, 1997). Therefore, it is most likely that leptin actually regulates lipolysis by controlling the activity of hormone-sensitive lipase. This enzyme is

controlled by cellular levels of cyclic AMP (cAMP) and although the regulation of lipolysis by leptin at the molecular level has not yet been fully described, preliminary evidence suggests that leptin, like glucagon and catecholamines, stimulates lipolysis by increasing cAMP concentrations (Takekoshi *et al.*, 1999).

Leptin-induced changes in lipid metabolism have also been measured in other tissues that store triacylglycerol. Leptin treatment of isolated pancreatic islets of rats causes an increase in fatty acid oxidation and a decrease in esterification, resulting in a reduction in intracellular triacylglycerol content (Shimuzu *et al.*, 1997). No change in triacylglycerol content, fatty acid oxidation or esterification are observed in the strain with the OB-R isoform mutation (*fa/fa*). Pancreatic islets of *fa/fa* rats contain as much as twenty times the amount of triacylglycerol found in lean rats (Lee *et al.*, 1997; Shimuzu *et al.*, 1997). Rats lacking a functional leptin receptor exhibit significantly higher expression of acyl-CoA synthetase and glycerol-3-PO<sub>4</sub> acyltransferase, two enzymes required for lipogenesis, and reduced expression of acyl CoA oxidase and carnitine palmitoyl transferase I, two enzymes involved in fatty acid oxidation. Because of these differences in enzyme expression, as well as the much higher lipid content of *fa/fa* non-adipocytes, it has been hypothesized that one of the functions of leptin is to keep the triacylglycerol content of non-adipocyte cells low, essentially limiting triacylglycerol storage to adipocytes (Unger *et al.*, 1999). In fact, triacylglycerol content of liver, skeletal muscle and pancreas can actually be decreased by *in vivo* adenovirus induction of hyperleptinemia (Shimabukuro *et al.*, 1997). Maintaining low concentrations of triacylglycerol in non-adipocytes is important as hyperlipidemia can result in lipotoxicity and apoptosis,

conditions that can lead to diabetes and other disease (Shimabukuro *et al.*, 1998; Unger *et al.*, 1999).

Finally, this study shows that leptin contributes in another way to increase energy expenditure by increasing the cycling rate of a futile substrate cycle. Increased substrate cycling has previously been shown to be a source of increased energy expenditure (Bahr *et al.*, 1990; Clark *et al.*, 1973a; Clark *et al.*, 1973b; Romijn *et al.*, 1993; Vallerand *et al.*, 1999; Wolfe *et al.*, 1987a; Wolfe *et al.*, 1990), therefore, it is not surprising that a substrate cycle also contributes to an increase in energy expenditure when stimulated by leptin. In this study, the increase in fatty acid reesterification accounted for 15% of the difference in energy expenditure that was observed with leptin treatment. Similarly, an increase in TAG/FA substrate cycling was calculated to account for approximately 11% of the increase in metabolic rate following burn injury (Wolfe *et al.*, 1987a).

Substrate cycles are constantly running in our bodies and there is an increasing number of studies that show they can significantly contribute to the total energy expenditure of individuals. This leads one to speculate that individual variation in their baseline cycling rates, or varying sensitivity to hormones (*e.g.* leptin), may contribute to the metabolic set-point or metabolic efficiency of individuals and, therefore, their energy use and storage. *In vitro* studies indicate that adipocytes of obese or previously obese humans generate less heat than adipocytes of lean humans (Nilsson-Ehle and Nordin, 1985; Sorbris *et al.*, 1982), while another *in vitro* study has reported decreased cycling of the TAG/FA substrate cycle in adipocytes of obese humans compared to lean humans (Bottcher and Furst, 1997). Also, Wolfe *et al.* (1987b) measured *in vivo* rates of this substrate cycle in lean (16% body fat) and obese (43% body fat) humans. Expressed per

kg of fat mass, they found that the lean subjects had a TAG/FA cycling rate that was 64% higher than the overweight subjects. This lends further support to the idea that futile substrate cycles may play a role in determining the overall metabolic efficiency of individuals, and possibly, their propensity towards obesity.

## **CHAPTER 6**

### **GENERAL CONCLUSION**

## *Summary*

The main purpose of my thesis was to identify the temporal changes in *in vivo* lipid mobilization and oxidation that occur in mammals during long-term fasting, arousal from hibernation, and following acute leptin treatment, and to attempt to elucidate the various mechanisms of action. Two different species were required in this thesis: the woodchuck and the laboratory rabbit. The woodchuck was used as an experimental model of a fasting adapted animal because they seasonally fast for the entire winter in a hypometabolic state and also in the spring at euthermic temperatures, while the NZW rabbit was used as the experimental model of a non-fasting adapted animal.

First, I determined the differences in rates of fuel oxidation and energy expenditure that occur in woodchucks fasting at euthermic temperatures at different times of the year and compared them with fasting rabbits (Chapter 2). This study was important because no information exists dealing with how hibernators, the most extremely adapted to fasting of all the mammals, change their rates of energy expenditure or fuel oxidation when fasting at normal temperatures. This study helps explain the metabolic adaptations that hibernators have to help them endure the normothermic fast they undertake following their final arousal from hibernation.

Second, I measured the rates of lipolysis and lipid mobilization in woodchucks that had just aroused from hibernation and the contribution of fatty acid reesterification to their total energy expenditure at this time (Chapter 3). This section of the thesis is physiologically significant because it is the first study to ever examine the possibility that a

hibernator may up-regulate the cycling rate of a substrate cycle as a mechanism to produce heat during arousal from hibernation. Almost all other research to date has focused on brown fat as the primary source of endogenous heat during this arousal. This study is also notable because it is the first to report *in vivo* rates of lipolysis and fatty acid mobilization in a hibernator.

Third, I measured the rates of fatty acid reesterification, lipolysis and lipid mobilization in rabbits fasting for an extended period of time to see if any temporal changes occurred as energy expenditure dropped (Chapter 4). Very few studies measure *in vivo* rates of lipolysis, fatty acid mobilization and reesterification because of the various difficulties associated with the techniques needed to measure them. Of the investigators that do these types of measurements almost all are human physiologists, so even fewer studies look at the effects of food deprivation. For these reasons there are only a couple of studies that have looked at any changes that occur in *in vivo* lipid metabolism with fasting, of which none involved a long-term fast (>5% loss of body weight). Therefore, the information in this section of the thesis is very meaningful because it examines the differences that exist in mammalian lipid metabolism between short and long-term fasting.

Finally I measured the effects that leptin has on lipolysis and lipid mobilization, and whether or not this hormone increases fatty acid reesterification as a mechanism to increase energy expenditure (Chapter 5). This section of the thesis is probably the most important because for the first time it details the effects that leptin has on these *in vivo* rates of lipid metabolism. Leptin is probably the most important hormonal regulator of mammalian lipid storage and disposal, so the information in this section is of important physiological significance. The novel physiological link between leptin, substrate cycling,

and total energy expenditure uncovered in this chapter may actually prove to be fundamental in the natural prevention of obesity.

The following general conclusions can be drawn from my thesis:

1. Woodchucks fasting in the summer lower their energy expenditure and protein oxidation as soon as a fast is initiated, however, woodchucks fasting in the spring do not, as their metabolic rate and protein utilization is already low. This is probably an adaptive survival mechanism as wild woodchucks normally have to fast at euthermic temperatures in the spring for extended periods of time following their final arousal from hibernation.
2. Woodchucks, as with most fasting adapted mammals, utilize very little protein when fasting, rely almost exclusively on adipose tissue. The rabbit, a non-fasting adapted animal, uses a greater proportion of protein while fasting although they do exhibit metabolic depression.
3. Compared to the rates measured in fasting woodchucks and rabbits, the rate of woodchuck TAG/FA substrate cycling following arousal from hibernation is extremely high. As a result, the contribution of this substrate cycle to total energy expenditure during this time is one of the highest that has been measured in any animal, decreasing the longer an animal has been aroused from hibernation.

4. In order to sustain the high cycling rate of the TAG/FA substrate cycle, the lipolytic rate of woodchucks following arousal from hibernation is one of the highest that has been measured in any animal. It is possible that that this rate of lipolysis is even higher when they are actually arousing.
5. As in previous studies on short-term fasting, the rates of appearance of glycerol and fatty acids initially double in mammals. However, this thesis demonstrates that the rates of glycerol and fatty acid appearance can actually decrease to pre-fasting levels with long-term fasting. It is likely that the metabolic depression induced with prolonged fasting accounts for these changes as less substrate is required for  $\beta$ -oxidation.
6. A reduction in the rate of fatty acid reesterification with long-term fasting can account for a small amount of the metabolic depression that accompanies a fast.
7. Acute leptin treatment causes increases in *in vivo* rates of lipolysis and fatty acid mobilization. Leptin administration also induces a shift in fuel utilization by reducing the relative contribution of carbohydrates and increasing the relative contribution of lipids to total energy expenditure.
8. Leptin stimulates the cycling rate of the TAG/FA substrate cycle, which in turn, can account for up to 15% of the increased energy expenditure also attributed to leptin treatment.

This study uncovers a novel physiological link between leptin, substrate cycling, and total energy expenditure that may prove fundamental in the natural prevention of obesity.

### *Future areas of research*

The high contribution of the TAG/FA substrate cycle to the energy expenditure of woodchucks aroused from hibernation is very unique. Most research on the mechanisms used by hibernators to generate heat during arousal from hibernation has been focused on uncoupling of brown fat mitochondria. This thesis demonstrates that substrate cycles may also play a critical role in thermogenesis during arousal. A subsequent study measuring the cycling rate of the TAG/FA substrate cycle during arousal from hibernation and not just following arousal is critical for establishing the thermogenic importance of this substrate cycle. It would also be interesting to look at the contribution of other substrate cycles to heat generation during arousal.

Much of this thesis focused on the changes that occur in lipid metabolism with either fasting or leptin treatment. Leptin treatment and short-term fasting were shown to have similar effects on *in vivo* lipid metabolism: both caused an increase in rates of lipolysis and fatty acid mobilisation. However, whether or not there is a relationship between leptin and lipid metabolism during fasting is still not clear. Independent of changes in mass, circulating leptin levels actually decrease with fasting or caloric restriction (Ahima et al., 1996; Ahren et al., 1997; Dubuc et al., 1998). Further study is needed to elucidate whether or not the drop in leptin is involved in any of the temporal

changes that occur in mammalian lipid metabolism during prolonged fasting. For example, it is possible that the decrease in leptin concentration observed with fasting may also contribute to the decline in energy expenditure that also occurs with fasting. This could be either through the effects of leptin on thyroid function and uncoupling proteins (Legradi et al., 1997), or through its effects on substrate cycling that was demonstrated in this thesis. However, because lipolysis, fatty acid mobilization and oxidation initially increase during a fast so it is still not clear how or if the decline in leptin concentration is related to these changes.

The chapter in this thesis dealing solely with leptin contains the first measurements of the effects of treatment with this hormone on *in vivo* rates of lipolysis, fatty acid mobilization and substrate cycling. Future studies could utilize doses in the physiological range, over different time frames and under different metabolic conditions (*e.g.* with fasting or hibernation). Preliminary evidence already shows that leptin is probably involved in the seasonality of energy expenditure and adiposity of hibernators and other seasonal mammals. For example, Klingenspor *et al.* (1996) found that leptin gene expression of Djungarian hamster adipose tissue was greatly reduced during the winter or during exposure to a short photoperiod. The decrease that is observed in leptin expression with decreasing photoperiod might be necessary so that changes in energy expenditure and appetite can occur. This is supported by studies that show that treatment of ground squirrels with leptin just prior to or following hibernation causes a reduction in normal food intake and weight gain (Boyer *et al.*, 1997; Ormseth *et al.*, 1996). Therefore, seasonal decreases in circulating leptin concentration, possibly coupled with decreases in leptin receptor density, probably help expedite the accumulation of fat mass that occur in hibernators especially prior to

entering hibernation. It has also been proposed that other factors, probably hypothalamic in origin, may override leptin's signals that would normally accompany weight gain (Mercer, 1998). In brief, the role of leptin in seasonal fat accumulation, as well as its role in the changes in metabolism that are observed during fasting and during hibernation, will all be fascinating areas of future study.

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