



**THE REGULATION OF FOOD INTAKE, BODY  
FAT STORES AND ENERGY BALANCE IN THE  
MARSUPIAL *SMINTHOPSIS CRASSICAUDATA***

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## THESIS SUMMARY

This thesis presents studies relating to the regulation of appetite, body fat stores and energy balance in the marsupial *Sminthopsis crassicaudata*. All of the studies presented have been published in international journals ( $n = 5$ ), accepted for publication ( $n = 2$ ) or submitted for publication ( $n = 3$ ).

The regulation of energy balance is a complex process that serves primarily to maintain body composition. Although studies have demonstrated differences in the regulation of energy balance across a variety of mammalian species, there is substantial evidence that fat mass is physiologically regulated, and that there are close relationships between fat storage, energy expenditure and food intake. In marsupials, however, the majority of studies have been genetic, ecological or reproductive, and there is very little information about the regulation of energy balance, food intake or body composition. In addition, studies of the regulation of energy balance in marsupials are of interest in an evolutionary context because of the early separation of the marsupial and eutherian lineages, estimated to have occurred ~ 150 million years ago. This ancient separation can be exploited to advantage in the study of homologous traits that have evolved independently from one another for millions of years, and may therefore complement studies done with more conventional laboratory mammals. Therefore, the broad aim of these studies was to evaluate the regulation of body fat stores, food intake and energy balance in the marsupial *Sminthopsis crassicaudata*.

Initial studies established the feeding behaviour of *S. crassicaudata* in the laboratory. It was demonstrated that *S. crassicaudata* consumed approximately 80% of daily intake during the dark phase, with 20-40% of total intake occurring during the first 3 hours of darkness. In addition, effects of both gender and photoperiod on food intake were demonstrated. No differences in body weight or tail width were observed between groups of males and females housed on long- and short-day photoperiods. Short-day housed females, however, showed reduced food intake as compared to short-day housed males, and long-day housed males and females. Following food deprivation for periods of 24 and 36 hours, both males and females housed on long- and short-day photoperiods showed a subsequent increase in food intake as compared to non-fasted controls, indicating compensatory post-fast hyperphagia.

The standard laboratory diet fed to *S. crassicaudata* had a caloric content of 1.01 kcal/g and contained 20% fat, 25% protein and 55% carbohydrate by dry weight. When fed with the higher calorie (2.99 kcal/g), higher fat (30%) and higher protein (60%) diet of mealworms, *S. crassicaudata* showed an increase in fat stores as compared to animals fed with the standard laboratory diet.

*S. crassicaudata* is one of a number of small animals that has caudal (tail) fat storage. Studies have demonstrated that an average of 25% of total body fat is stored in the tail of *S. crassicaudata*. In addition, *S. crassicaudata* has no intra-abdominal fat and all other fat is deposited sub-cutaneously. Tail fat stores are proportional to total adiposity and alterations in tail fat can be used to monitor changes in total body fat stores. Tail removal (approximately a 25% lipectomy) induced re-accumulation of fat



in sub-cutaneous adipose tissue depots, without changes in food intake, suggesting that body fat stores are defended by alterations in metabolic efficiency or decreased physical activity.

The *ob* protein leptin, circulates in proportion to adipose tissue stores and is believed to signal to the brain the size of these adipose tissue stores. Leptin is involved in the control of body fat stores through coordinated regulation of feeding behaviour, metabolism, energy balance and neuroendocrine responses. The *S. crassicaudata* leptin gene was cloned and mapped to chromosome 5. The mature protein was shown to be 82% similar to both mouse and human proteins, and northern blot analysis showed expression of leptin mRNA only in white adipose tissue. This study provided the first molecular information on leptin from a marsupial species. The function of leptin to decrease fat mass and appetite appears to be conserved across species; as in rodents, *S. crassicaudata* fed standard laboratory diet decreased body weight, tail width (fat mass) and energy intake in response to chronic peripheral administration of human recombinant leptin. However, when fed both standard laboratory diet, together with the higher fat and calorie, preferred diet of mealworms, leptin administration failed to effect body weight, fat mass or total food intake in *S. crassicaudata*. The proportion of mealworms eaten was increased compared to saline treated controls. These data suggest that intake of a preferred diet with an increased dietary fat, protein and caloric content results in increased adiposity, associated with resistance to the effect of exogenously administered leptin.

Plasma nutrient levels have been demonstrated to effect feeding in mammals. In *S. crassicaudata* as in other mammals, fasting induced a decrease in plasma glucose levels. However, neither insulin induced hypoglycaemia nor metabolic blockade of glucose utilisation with 2-deoxy-D-glucose, increased food intake in *S. crassicaudata*. These data suggested that mechanisms other than glucose availability are important in the regulation of food intake in this species. These observations, together with data from other species suggest that glucoprivation is not a highly conserved mechanism for signalling the need to increase food intake. For example, in birds, food intake is not increased in response to glucoprivation, but oxidation of fatty acids plays an important role. Fasting increases peripheral plasma free-fatty acid levels in *S. crassicaudata* in order to maintain energy supply to the rest of the body. Fasting is also associated with an increase in food intake. Administration of mercaptoacetate, an inhibitor of fatty acid oxidation, however, did not affect food intake in *S. crassicaudata*. The role of fatty acid metabolism in the regulation of food intake in *S. crassicaudata* therefore remains unclear, but presumably there are mechanisms by which alterations in nutrient utilisation can be sensed.

Numerous hypothalamic and gastrointestinal peptides have been implicated in the control of feeding, however their effects on appetite in marsupials have not previously been determined. Peptides found within the hypothalamus, including corticotrophin releasing factor (CRF) and urocortin (UCN), decrease food intake and increase energy expenditure when administered both centrally and peripherally to rodents, although UCN has a more potent anorectic effect than CRF. The amino acid sequence of CRF demonstrates a high level of homology among species suggesting

conservation of physiological function. The effects of CRF and UCN on food intake in marsupials are not known. As in other mammals, peripherally administered CRF induced cortisol release in *S. crassicaudata* via the CRF1 receptor and central CRF administration potently decreased food intake. Both peripheral CRF and UCN decreased food intake in *S. crassicaudata*. Large doses of CRF were required to decrease food intake when administered peripherally and UCN was considerably more potent (approximately 50 fold) in this regard. The anorectic effects of CRF and UCN were not blocked by a CRF1 receptor antagonist, suggesting that when administered peripherally the effects of CRF and UCN on food intake are mediated primarily by the CRF2 receptor.

Endogenous opioid peptides activate food seeking behaviour and influence macronutrient choice in a number of animal species and previous studies have suggested that the palatability of food is strongly modulated by the opioid feeding system. The  $\mu$  opioid receptor antagonist naloxone and the  $\delta$  antagonist naltrindole decreased the intake of laboratory diet in *S. crassicaudata*, whereas the  $\kappa$  antagonist nor-BNI was without an effect. Studies have shown that *S. crassicaudata* will eat mealworms in preference to the normal laboratory diet. The effect of naloxone to decrease food intake was enhanced in *S. crassicaudata* fed both laboratory diet and mealworms, due to a preferential suppression of the intake of mealworms. These studies demonstrate that endogenous opioid peptides influence food intake and food choice in *S. crassicaudata* and that the role of the opioid feeding system is, at least in part, modulated by food palatability. In *S. crassicaudata*, opioid effects on feeding appear to be mediated by  $\mu$ , rather than  $\kappa$  or  $\delta$  opioid receptor pathways.

Gastrointestinal peptides, released from the gut in response to food intake have been demonstrated to decrease feeding in mammals, although these effects are species specific and photoperiod dependent. The effect of GI peptides on feeding has not been determined in marsupials. Unlike CRF and UCN, peripheral cholecystokinin, bombesin and glucagon-like peptide-1 administration, at doses shown to effect feeding in rats, had no effect on food intake in *S. crassicaudata*. Gastrin releasing peptide induced a small decrease in food intake in short- but not long-day housed animals.

In rodents, changes in body fat stores may result from alterations in energy expenditure and/or the fuel mix oxidised, and there is evidence that disturbance of these systems may lead to obesity. Many mammals rely on brown adipose tissue (BAT) for the production of heat by non-shivering thermogenic mechanisms during cold exposure and re-warming from hibernation. Heat is generated intracellularly by the uncoupling of oxidative phosphorylation, regulated by uncoupling protein-1 (UCP1), and UCP1 is expressed only in BAT. UCP2 is a recently described uncoupling protein with 59% homology to UCP1. UCP2 has a wide tissue distribution, and may have a primary role in regulating nutrient partitioning (FFA utilisation), rather than a major thermogenic role. The importance of thermogenesis in regulating fat stores in mammals, particularly those where BAT is of lesser importance, remains to be determined. The presence of BAT in marsupials is controversial and UCP1 expression has not been previously demonstrated. *S. crassicaudata* is known to use torpor as an energy conserving mechanism. However, studies presented in this thesis have shown that this marsupial displays a remarkable

plasticity in its ability to vary body temperature without entering torpor, when the environmental temperature is moderately decreased. In addition, based on electron-microscopic characteristics, GDP binding and immunological detection of mitochondrial uncoupling protein 1 (UCP1) in white adipose tissue, these studies have identified the presence of BAT in the interscapular fat pads of *S. crassicaudata*. This is the first unequivocal evidence of BAT in a marsupial, although as in humans it does not appear to be used for the production of heat, and it is not used for re-warming from torpor. In addition to UCP1, *S. crassicaudata* expresses the closely related UCP2 in a similar tissue distribution to other mammals.

The relationship between changes in photoperiod and alterations in gonadal steroids and the subsequent effects on body composition are complex and differ between species. Studies presented in this thesis have demonstrated that in *S. crassicaudata* fat mass increases in response to both short photoperiod and ovariectomy (OVX), without significant alterations in energy intake, suggesting increased metabolic efficiency. The effects of photoperiod on fat mass in *S. crassicaudata* are mediated by both gonadal steroid dependent and independent mechanisms. Although UCP2 expression was not effected by photoperiod, ovariectomy decreased UCP2 expression in muscle. Alterations in UCP2 expression may therefore mediate energy adjustments that occur in response to ovariectomy. It is likely that changes in UCP2 mRNA expression in muscle but not fat are associated with changes in energy balance, suggesting that UCP2 may have a role in the maintenance of body composition by regulating thermogenesis, nutrient partitioning or both.

These studies have provided novel data on the regulation of food intake, body fat stores and energy balance in the marsupial *Sminthopsis crassicaudata*, representing fundamental advances in marsupial biology.

## STATEMENT OF ORIGINALITY

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed:

Date: 25/11/00

Perdita Jane Hope

## **DEDICATION**

**This thesis is dedicated to my parents Rae and Rory Hope for their unfailing love, support and friendship. Without them this achievement would not have been possible.**

**Thankyou!!**



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## CHAPTER 1

# THE REGULATION OF BODY WEIGHT

## 1.1 INTRODUCTION

The regulation of body weight and fat mass in mammals is poorly understood, largely because the interactions between energy intake and energy expenditure are complex. The majority of studies examining these processes, however, have been performed in rodents, and there is little information available from other eutherian species. In addition, there is comparably little information regarding the regulation of body fat stores, food intake and energy balance in marsupials. Marsupials and eutherians are thought to have last shared a common ancestor approximately 130-150 million years ago. Any conservation of mechanisms involved in energy intake and expenditure in species as divergent as marsupials and rodents, may imply that such mechanisms are highly conserved and are therefore also likely to be involved in regulating energy balance in humans. This chapter reviews current knowledge of the factors involved in the regulation of both food intake and energy expenditure in mammals.

## 1.2 THE 'SET-POINT' THEORY OF BODY WEIGHT

There is substantial evidence that fat stores are physiologically regulated, with energy balance being maintained by a system that continually matches energy intake and expenditure (Chlouverakis & Hojnicky 1974, Liebelt *et al* 1965, Schemmel *et al* 1971). In the majority of mammals, including humans, body weight remains stable for prolonged periods of time; coordinated adjustments of energy balance (intake and expenditure) serve to stabilise the weight of individuals around a 'set-point', and resist displacement from this level, Fig 1.1.

### 1.2.1 *The concept of a 'set-point' for body weight*

#### 1.2.1.1 *Animal studies*

The 'set-point' theory of body weight has been supported by numerous studies in animals (Fantino & Cabanac 1980, Harris *et al* 1986, Hunsinger & Wilson 1986, Keesey *et al* 1976, Stricker 1978). For example, if rodents are made obese (eg. by force feeding) and then returned to their normal *ad libitum* diet, an increase in energy expenditure, and a decrease in energy intake is observed. Consequently, the animal returns to its previous weight (Stricker 1978). Conversely, a loss of fat stores (eg. due to starvation) is associated with a subsequent decrease in energy expenditure, and increases in appetite, energy intake and body weight, once the animal is again allowed to eat freely (Stricker 1978). In rats, lesions of the lateral hypothalamus decrease body weight; this 'reduced' body weight is maintained and defended at the new level, with comparable results to non-lesioned animals (Keesey *et al* 1976). Anorectic drugs (eg. fenfluramine) may reduce the body weight 'set-point' in rodents, with food

consumption being affected secondarily to achieve a new body weight (Hunsinger & Wilson 1986, Stunkard 1982). This reduction in body weight is not associated with tolerance to the anorectic drug; when treatment is ceased there is a return to initial body weight.

#### 1.2.1.2 *Human studies*

Although evidence from animal studies strongly supports the concept of a body weight 'set-point' (Fantino & Cabanac 1980, Harris *et al* 1986, Hunsinger & Wilson 1986, Stricker 1978), studies on the regulation of body weight in humans are difficult to perform (Stunkard 1982). In humans, erratic fluctuations in body weight do occur (as in other mammals), reflecting daily alterations in fluid balance, variations in calorie and macronutrient intake, and alterations in levels of energy expenditure. In adult humans, variations in body weight in excess of  $\pm 5$  kg over a 3 year period are not uncommon (Forbes *et al* 1986). Despite this, the regulation of body fat stores in humans is reasonably precise. It has been estimated that an average 70 kg male would need consume about 900 000 calories per year to maintain a constant body weight. If the physiological system that maintains body weight has an annual error of 3% (*ie.* 27 000 calories), body weight could potentially vary by 4.5 kg per year. In reality however this rarely occurs, and in most individuals body weight only increases by approximately 9 kg from the age of 25 to 55 (Weigle 1994). Therefore, the annual error is only about 0.15%. It is unlikely that body weight could be maintained so precisely without the existence of a physiological system acting to regulate body fat stores around a 'set-point'. This concept is supported by observations in healthy humans that increased fat deposition resulting from over-feeding induces an increase

in energy expenditure and a decrease in energy intake, resulting in weight loss (Diaz *et al* 1992, Roberts *et al* 1990). Conversely, a loss of fat stores due to fasting induces a decrease in energy expenditure and an increase in appetite and energy intake (Weigle *et al* 1988).

### 1.2.2 *Body weight 'set-points' of obese individuals*

Obese animals and humans display behavioural and metabolic adjustments to weight perturbations. In the obese, the mechanisms that regulate body weight are not defective, and operate similarly to those of lean animals and humans. These mechanisms, however, are maintained to defend abnormally high body weight 'set-points' (Keesey & Corbett 1984, Keesey *et al* 1976). Obese mammals actively resist efforts to reduce weight. For example, in the obese Zucker rat the responses to food deprivation and weight loss are comparable to normal weight controls (Keesey & Corbett 1984).

### 1.2.3 *Does a body weight 'set-point' exist in growing mammals?*

Young growing mammals exhibit a stable but 'dynamic set-point', with body weight and composition determined by chronological age (Wilson & Osbourn 1960). Growth of rats is restricted by limited food availability, however following return to *ad libitum* feeding, animals show a period of compensatory growth by increasing food intake or efficiency of energy utilisation, resulting in a return to control weight and body composition (Wilson & Osbourn 1960). In addition, when young (3wk) rats are overfed, there is an increase in both fat and lean mass. Following cessation of overfeeding, animals become hypophagic for a short period. Body weight

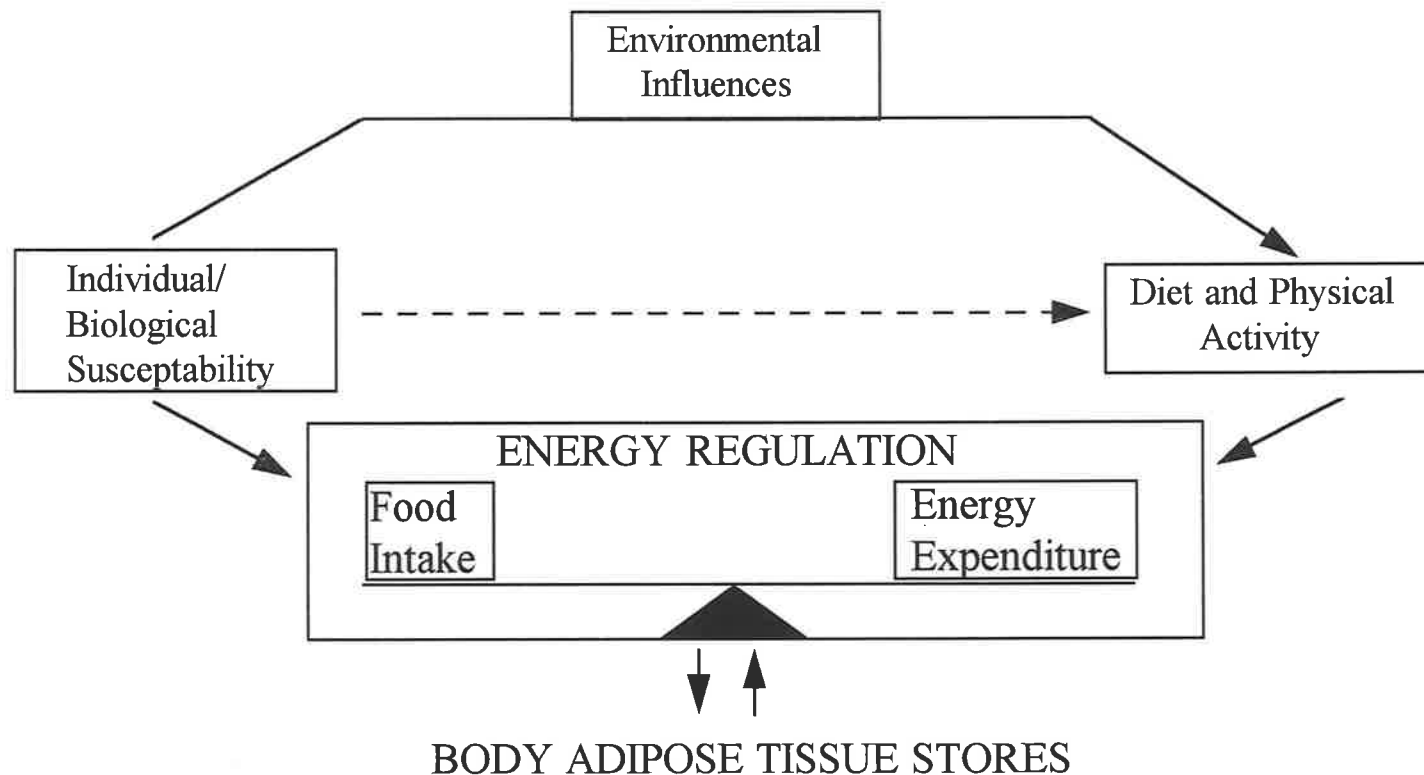


**Figure 1.1:** Model for the regulation of energy balance and adiposity. Body fat stores are maintained at a ‘set-point’ as a result of the balance between energy expenditure and food intake, which are in turn influenced by a range of factors, including individual and biological susceptibility, environmental effects, diet and physical activity.

continues to increase slowly but body fat stores decrease to that of control animals (Drewry *et al* 1989). Rodent studies therefore suggest that the mechanisms for regulating body weight, body composition and energy balance in mammals are operative during growth (Drewry *et al* 1989, Harris 1990, Wilson & Osbourn 1960).

#### 1.2.4 *The 'unsettling mythology of set-points'*

The 'set-point' theory is not universally accepted, partly because of the difficulties in performing 'set-point' studies, particularly in humans (Garn 1992, Garrow & Stalley 1975, Harris 1990). There is also evidence against a precise system for the regulation of body weight. For example, following short-term starvation, the body weight of rats do not reach those of controls (Armstrong *et al* 1980). In contrast, rats can be made obese by feeding them a highly palatable high fat diet (Wirtshafter & Davis 1977). In addition, the observed increases in fat stores of some birds and mammals before migration and hibernation, and the increase in body weight during pregnancy, all suggest that the 'set-point' of body weight is labile (Morley & Levine 1983).

Powerful environmental factors influence both energy intake and expenditure, and may overwhelm the physiological regulatory mechanisms utilised in the regulation of body weight. Susceptibility to these environmental factors is affected by genetic and other biological forces such as sex, age and hormonal activity. Changes in diet and/or physical activity may also contribute to alterations in body fat stores, Fig 1.1. For example, epidemiological studies in humans have established that the prevalence of obesity in Western society and developing countries is increasing (Prentice & Jebb 1995). This suggests that the high fat diets associated with a 'modern day' lifestyle

can over-ride satiety mechanisms, and in combination with the sedentary lifestyles of these countries, may contribute to weight gain (Prentice & Jebb 1995, World Health Organization 1997).

Because a variety of genetic, dietary and environmental factors combine to determine the steady state of weight maintenance, fat mass does not appear to be under strict 'set-point' control (Jequier & Tappy 1999). Consequently, the term 'unsettling mythology of set-points' has been used (Morley & Levine 1983). Based on the available evidence, it may be more reasonable to define the 'set-point' of body weight and fat mass as a 'settling-point', subject to environmental and other factors.

### **1.3 THE LIPOSTATIC HYPOTHESIS**

There is relatively little variation in protein and carbohydrate stores in adult mammals, and alterations in body weight are, therefore, primarily the result of changes in adipose tissue stores. The lipostatic hypothesis proposed by Kennedy (1953) suggests that the amount of fat in the body is measured and sensed by neural or hormonal signals that are relayed to the brain, which in turn adjusts energy balance.



### 1.3.1 *Evidence supporting the lipostatic hypothesis*

#### 1.3.1.1 *Lipectomy studies*

If fat mass is physiologically regulated, and a signal proportional to total adipose tissue mass is monitored by the brain to control energy balance, lipectomy (surgical removal of adipose tissue) should potentially result in increased food consumption and/or reduced energy expenditure, in order to restore fat mass.

Several studies have examined the effect of lipectomy on subsequent adipose tissue mass in mammals. After moderate lipectomy, the Syrian hamster (*Mesocricetus auratus*) (Hamilton & Wade 1988), Siberian hamster (*Phodopus sungorus*) (Mauer & Bartness 1994) and the golden-mantled ground squirrel (*Spermophilus lateralis*) (Dark *et al* 1985) all have the capacity to increase their fat mass to previous levels. For example, following lipectomy (75-85% of inguinal subcutaneous, epididymal and retroperitoneal fat depots), ground squirrels restore adipose tissue mass in subcutaneous and retroperitoneal fat depots (Dark *et al* 1985). This occurs without an increase in food intake, suggesting that the additional energy required for restoration of fat mass was derived from reduced energy expenditure (eg. possibly due to decreased activity and/or non-shivering thermogenesis) (Dark *et al* 1985). In Syrian hamsters, removal of dorsal, inguinal, subcutaneous, parametrial and retroperitoneal fat pads is followed by complete restoration of total body fat mass to the level of the sham controls (Hamilton & Wade 1988).

Similar studies performed in rats have produced comparable but more variable results, dependent on: i) the age of the animal, ii) the strain (animals with genetic or acquired

obesity syndromes show a more rapid and complete response) (Chlouverakis & Hojnicky 1974, Liebelt *et al* 1965), iii) gonadal function (Faust *et al* 1984), and iv) dietary fat content (Faust *et al* 1977a). Liebelt *et al* (1965) have shown that surgical removal of adipose tissue results in enlargement of the remaining fat depots in rats. The clearest effects were demonstrated in genetically obese animals, or animals with damage to the ventromedial hypothalamus induced by gold-thio-glucose. Some studies in rats have failed to find an increase in remaining adipose tissue depots following lipectomy (Faust *et al* 1976, Kral 1976).

In many mammals, the restoration of fat mass following lipectomy does not appear to be associated with increased food intake (Weigle 1994). For example, some studies in obese mice have failed to observe an increase in feeding following lipectomy (Schemmel *et al* 1971). Small adjustments in food intake could, however, be obscured by post-operative inflammation and wound healing. Many lipectomy studies have been criticised for the degree of trauma that the surgical procedure may have caused, thereby confounding results. In this respect, a lipectomy study in mice by Liebelt *et al* (1965), in which the surgical procedure was less extensive, was associated with an increase in food intake. The reason variable results is unclear, however it is possibly due to differences in the amount and location of adipose tissue removed, the species of animal tested, or the way measurements were taken.

The results of lipectomy (liposuction) studies in humans, although conflicting, have provided some evidence to support the lipostatic hypothesis. Short term follow up studies (1-2 months post operatively) suggest that surgical fat removal does not result

in compensatory changes in metabolic efficiency or fat cell size and distribution, in 7 previously weight stable women (Lambert *et al* 1991). Long-term follow up was not reported for this study. However, previous studies demonstrated that lipectomy in humans may produce weight loss in the short to medium term, but weight is generally regained in the long term (Caterson 1990).

To date, the majority of lipectomy studies have been performed in rodents, and have required invasive and potentially traumatic surgical procedures. The overall regulation of body fat stores and the effect of lipectomy has not been adequately assessed in marsupials. It was hypothesised that, as in mammals, marsupials regulate total body fat around a 'settling-point'. The marsupial *Sminthopsis crassicaudata* is known to use its tail as an organ of fat storage (see section 2.4.5). Tail removal of *S. crassicaudata*, a quick and non-invasive procedure, could potentially be used to assess the effects of lipectomy in a marsupial species.

#### 1.3.1.2 *Parabiosis studies*

Parabiosis studies involve joining two animals such that fibrous tissue and vascular, but not neural, connections develop between them (Huff *et al* 1950). Circulating substances are transferred slowly (40-120 minutes) between the parabionts, and therefore transfer of short-lived circulating factors, such as gastrointestinal meal termination signals (eg. cholecystokinin, see section 1.6.3.2), does not occur (Huff *et al* 1950). The studies of Schmidt & Andik (1969) are consistent with this concept as they demonstrated that satiety induced in partners of experimentally obese parabiotic

rats, is related to the elevated body fat content of obese animals, rather than gastrointestinal responses to food consumption.

There are numerous studies reporting the effects of parabiotic experiments on body weight regulation. For example, Hervey (1959) demonstrated that the induction of obesity by lesions of the ventromedial hypothalamus in one animal of a parabiotic rat pair, induced hypophagia and weight loss in the un-lesioned rat. In partners of rats that are overfed by gavage for 46 days to induce obesity, there are decreases in food intake and fat mass (Harris & Martin 1984). Measurement of circulating insulin, growth hormone, glucose and corticosterone suggested that none of these factors were responsible for the loss of body fat observed in the lean rat parabionts (Harris & Martin 1984). Replacement of calories to the lean rat parabionts by tube feeding prevented their loss of body fat (Harris & Martin 1990). Studies by Chlouverakis (1972) found that in the obese *ob/ob* mouse, weight gain was attenuated by parabiosis with lean mice, supporting earlier studies by Hausberger (1958) who showed that non-obese mice attenuated the weight gain of obese littermates.

In 1973, Coleman performed what proved to be a classic series of experiments, demonstrating that a circulating factor produced by adipose tissue is responsible for the regulation of body fat via alterations in food intake (Coleman 1973). In these studies, the circulation of normal mice and mice with different forms of obesity (*ob/ob* and the *db/db* mice, both of which are obese, hyperphagic, hypothermic, lethargic, diabetic and infertile) were parabiosed. When the *ob/ob* mouse was parabiosed to a *db/db* mouse, the *ob/ob* mouse lost weight and died of starvation. When the *ob/ob*

mouse was parabiosed to a normal mouse, the *ob/ob* mouse ate less and lost weight. When the *db/db* mouse was parabiosed to a normal mouse, the normal mouse lost weight and died of starvation. From these experiments, Coleman concluded that the *db/db* mouse produced a circulating factor to which it could not respond, and that the *ob/ob* mouse was deficient in this factor (Coleman 1973, Coleman & Hummel 1969). More than 20 years later, Coleman's predictions were realised when, in 1994, the gene defect in the *ob/ob* mouse was identified, and the *ob* gene cloned (Zhang *et al* 1994). The *ob* gene codes for a protein called leptin, which is deficient in the *ob/ob* mouse. The *ob/ob* mouse has 2 separate mutations in the *ob* gene resulting in either a premature stop codon, or the total absence of *ob* mRNA (Moon & Friedman 1997, Zhang *et al* 1994). It has now been shown that the abnormality in the *db/db* mouse is a defective leptin receptor (Tartaglia *et al* 1995). Leptin is therefore the circulating factor postulated by Coleman (1973). Exogenously administered leptin restores the abnormalities of the *ob/ob* mouse, and produces weight loss and decreased food intake in lean mice. Plasma leptin levels are highly correlated to adipose tissue stores, and levels of *ob* gene expression correlate with body fat content (Considine *et al* 1996, Frederich *et al* 1995b, Maffei *et al* 1995). Leptin and the *ob* gene are discussed further in section 1.5.

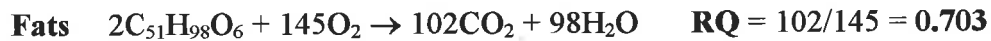
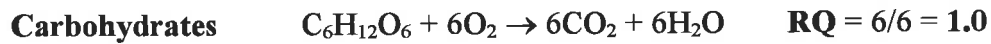
## **1.4 NUTRIENT PARTITIONING, MACRONUTRIENT METABOLISM, AND DIET COMPOSITION.**

The maintenance of body composition is dependent not only on balances between food intake and energy expenditure, but also on the ability of the body to oxidise a fuel mixture that is, on average, equivalent to the nutrient mixture consumed (Flatt 1987). This balance is achieved by interchangeably using the metabolic intermediates derived from carbohydrates, fats and proteins in order to regenerate ATP (provided that proteins provide at least 8-10% and essential fatty acids at least 1-2% of total energy) (Flatt 1996). Nutrient partitioning describes shifts in the flow of nutrients between utilisation for energy or storage as protein, fat or glycogen. The concept of nutrient partitioning arises primarily from studies showing that there is a hierarchy in the extent to which macronutrients are stored or oxidised (Flatt 1988).

### **1.4.1 *Substrate oxidation***

Substrate oxidation is determined by the need to regenerate ATP for use in metabolic functions, temperature regulation and activity (Flatt 1995a,b). The respiratory quotient (RQ) provides a measure of the relative rates of carbohydrate and fat oxidation, and is determined by the ratio of CO<sub>2</sub> produced to O<sub>2</sub> consumed. RQ varies between 0.7 and 1.0, depending on the relative proportions of carbohydrate and fat being oxidised. The RQ of carbohydrate is approximately 1.0 and that of fat approximately 0.7. This is because O<sub>2</sub> and H are present in carbohydrate in the same proportions as water, whereas for fat, additional O<sub>2</sub> is necessary for the formation of H<sub>2</sub>O (Ganong 1985).

*i.e.*



Since protein makes a constant but minor contribution to the substrate mix which is oxidised, the RQ is not affected substantially by protein oxidation. An average RQ of 0.82 has been calculated for protein, although its determination is a complex process (Ganong 1985).

The food quotient (FQ) describes the ratio of  $\text{CO}_2$  produced to  $\text{O}_2$  consumed during the biological oxidation of a representative sample of the diet. Comparison of RQ to the FQ of a particular diet can be used to determine whether the fuel mix being oxidised contains more or less fat than is present in the diet. An RQ/FQ ratio greater than 1 indicates a positive energy balance, and an RQ/FQ ratio less than 1 indicates a negative energy balance (Flatt 1996, Ganong 1985). Therefore, in order to maintain energy balance, an organism must burn a fuel mixture with an average RQ that matches the FQ of the diet. The relationship between RQ and FQ is, however, only a conceptual tool in understanding the interactions between dietary fat content, fuel metabolism, and body composition. It is difficult experimentally to show an RQ greater than FQ for minor energy imbalances that may eventually lead to accumulation of excess amounts of body fat (Flatt 1996).

Daily variations in food intake and physical activity result in deviations from equilibrated substrate balances. However, the utilisation of energy reserves occurs in

such a way as to minimise changes in protein content and to maintain glycogen concentrations within a given range (Abbott *et al* 1988). The fraction of dietary energy provided by protein is relatively small and constant, and the body maintains a constant protein content by adjusting amino acid oxidation to amino acid intake. Because of this, body weight maintenance is achieved primarily as a result of utilisation of carbohydrate and fat. The storage capacity for fat is larger than that of glycogen and protein (Flatt 1995a) and, in most cases, variations in overall energy balance are accommodated for by changes in fat stores. The hierarchy in the extent to which macronutrients can be stored thus determines their metabolic fate once ingested.

The composition of the fuel mix oxidised and the respiratory quotient are influenced by circulating substrate and hormone concentrations, which are in turn affected by nutrient intake (Flatt 1996). For example, the hormone insulin promotes storage of carbohydrate, amino acids and fat, inhibits fat mobilisation, and increases carbohydrate oxidation. When glucose absorption from the gut is completed, a decline in insulin secretion, and the release of catecholamines and glucagon, activate the mobilisation of glycogen and fat reserves to assure an adequate supply of glucose and free fatty acids in the circulation (Flatt & Blackburn 1974). An increase in fat stores is the physiological response to excess calories in the diet and there is some evidence that insulin resistance is a defence mechanism to prevent further increases in fat storage (Eckel 1992).



### 1.4.2 *Metabolism of macronutrients*

The energy value of common macronutrients measured in a bomb calorimeter, are 4.1 kcal/g carbohydrate, 9.3 kcal/g of fat and 5.3 kcal/g of protein. Within the body, oxidation of protein is incomplete; the end products of protein catabolism being urea and related nitrogenous compounds, as well as CO<sub>2</sub> and H<sub>2</sub>O. Therefore, the caloric value of protein in the body is only 4.1 kcal/g (Ganong 1985).

#### 1.4.2.1 *Protein*

Mammals are able to maintain a stable body protein content, and this occurs regardless of differences in the ratio of carbohydrate to fat in the diet (Flatt 1995a). Regulatory mechanisms minimise protein losses during food deprivation and prevent protein accumulation following consumption of high protein diets (Cahill Jr 1971). Most ingested proteins are digested and their constituent amino acids absorbed, while the body's own proteins are being continuously hydrolysed and re-synthesised (Flatt 1996). The amino acids formed from endogenous protein breakdown, together with those derived from ingested protein, form an 'amino acid pool' that supplies the needs of the body (Ganong 1985). Small gains or losses of protein influence amino acid oxidation, and induce corrective responses required to compensate for small day to day changes in nitrogen balance (Flatt 1995a).

#### 1.4.2.2 *Carbohydrate*

The principal product of carbohydrate digestion is glucose, whose storage product glycogen is present in most body tissues, particularly the liver and skeletal muscle. In mammals, glycogen stores are important in the maintenance of a stable blood glucose

concentration and for facilitating rapid access to energy, such as during sudden muscular responses (Flatt 1995b). The importance of glycogen stores in the physiological system has resulted in the evolution of regulatory mechanisms, including endocrine signals, giving high priority to the adjustment of carbohydrate oxidation to carbohydrate availability (Flatt 1995a). There is a net uptake of glucose by the liver when blood glucose is high, and a net discharge when it is low. With prolonged fasting, glycogen is depleted and there is increased gluconeogenesis (conversion of non glucose molecules to glucose) from amino acids and glycerol in the liver. The liver therefore acts as a 'glucostat', maintaining a constant circulating glucose level. Large carbohydrate intakes lead to high rates of carbohydrate oxidation, and glycogen concentrations return to normal (Ancheson *et al* 1984). Conversion of carbohydrate into fat allows an accumulation of fat reserves when the diet is low in fat. In humans, large carbohydrate loads are handled primarily by conversion to glycogen and the use of glucose as a fuel. Consumption of carbohydrate thus reduces the need to use fat as fuel and is therefore an important determinant of how much of the fat consumed will be retained (Flatt 1995a).

Maintenance of stable glycogen concentrations is facilitated by signals that regulate food intake when glucose concentrations and/or glycogen reserves rise or decline excessively (Mayer & Thomas 1967). In humans, hypoglycaemia induces an urge to find food, yet food intake among free living individuals is such that glycogen stores are maintained below the concentration at which rapid rates of *de novo* lipogenesis could be induced (Ancheson *et al* 1984, Hellerstein *et al* 1991). An increase or

decrease in the carbohydrate content of the diet leads to a decrease or increase in energy intake respectively (Kendall *et al* 1991).

There is strong evidence that glucoprivation is an important stimulus of food intake in many mammals including rats and monkeys (Houpt & Hance 1971, Smith & Epstein 1969), although there are species specific differences (Rowland 1978, Rowland *et al* 1985, and see chapter 8). Furthermore, glucoprivation does not increase food intake in birds (Boswell *et al* 1995, Hatfield 1972, Smith & Bright-Taylor 1974), suggesting that other metabolites such as free-fatty acids may play a more important role in food intake. The effect of glucoprivation on food intake has not previously been determined in a marsupial species.

#### 1.4.2.3 *Fat*

According to Flatt's hypothesis (1996), changes in the composition of the fuel mix oxidised allows compensation for temporary gains or losses of glycogen and proteins, at the expense of preventing effective control over fat balance. Fat oxidation is determined primarily by the difference between overall energy expenditure and the amount of energy provided by ingestion of protein and carbohydrate. Whereas stable protein and glycogen reserves can be maintained by appropriate changes in the rate of amino acid and glucose oxidation, deviations from equilibrated fat and energy balances can only be corrected by appropriate changes in food intake (Flatt 1995a). Short term errors in fat balance are too small to significantly affect the size of the body's fat stores, circulating fatty acid concentrations, fat oxidation, or food intake, but the cumulative effects of repeated imbalances between fat intake and fat oxidation

can lead in time to significant changes in the size of the adipose tissue mass (Flatt 1996). Both excess fat intake and reduced fat oxidation are factors that favour weight gain, and the regulation of dietary fat is therefore important in the maintenance of a stable body composition.

Inhibition of fatty acid oxidation has been demonstrated to increase food intake and result in weight gain in a range of mammals (Friedman & Tordoff 1987, and see chapter 9), although these effects are species specific, and diet and photoperiod dependant (Scharrer & Langhans 1986, Singer *et al* 1997, Stamper & Dark 1997). In birds, fatty acid utilisation is relied upon to increase food intake and fat storage before migration (Boswell *et al* 1995). The effect of inhibition of fatty acid oxidation on food intake in a marsupial species has not previously been studied.

## 1.5 LEPTIN, THE *OB* GENE AND THE REGULATION OF BODY WEIGHT

The *ob* gene, isolated by positional cloning from the *ob/ob* mouse, encodes a 167 amino acid hormone called leptin, that is expressed predominantly in white adipose tissue (Cinti *et al* 1997, Masuzaki *et al* 1995, Zhang *et al* 1994). Leptin expression has also been demonstrated in the stomach and placenta, albeit at low levels (Bado *et al* 1998, Masuzaki *et al* 1997). The crystal structure of leptin suggests that it belongs to the cytokine family (Friedman JM 1998, Madej *et al* 1998, Zhang *et al* 1997). Leptin circulates in both mouse and human plasma as a protein of 16K, as either a free

form, or bound to leptin-binding proteins (Halaas *et al* 1995). A 19K form of leptin has been identified in rat stomach, although the function and molecular nature of this form is not yet known (Stephens *et al* 1995).

Plasma leptin levels correlate closely with total adipose tissue stores (Considine *et al* 1996, Maffei *et al* 1995). In both mice and humans weight loss is associated with a fall in plasma leptin levels; in obese humans plasma leptin levels are increased (Maffei *et al* 1995). The levels of expression of the *ob* gene correlate with body fat content (Frederich *et al* 1995b, Maffei *et al* 1995). Leptin thus provides a communication link between fat tissue and the brain, acting on receptors found primarily in hypothalamic nuclei and signalling the size of adipose tissue stores to the central nervous system (Mercer *et al* 1996, Schwartz *et al* 1996b). Consequently, body fat stores are controlled through coordinated regulation of feeding behaviour, metabolism, neuroendocrine responses, the autonomic nervous system and energy balance, Fig 1.2.

### **1.5.1      *Rodent studies***

The *ob/ob* mouse is hyperphagic, obese, diabetic, hypothermic, hypoactive, infertile, and has a low resting metabolic rate (Coleman 1978). Peripheral or central administration of recombinant mouse *ob* protein decreases both food intake and body weight of *ob/ob* mice, as a result of increased energy expenditure and fat oxidation. Effects are seen with both acute and chronic leptin administration (Campfield *et al* 1995, 1996, Halaas *et al* 1995). Leptin administration also normalises the other

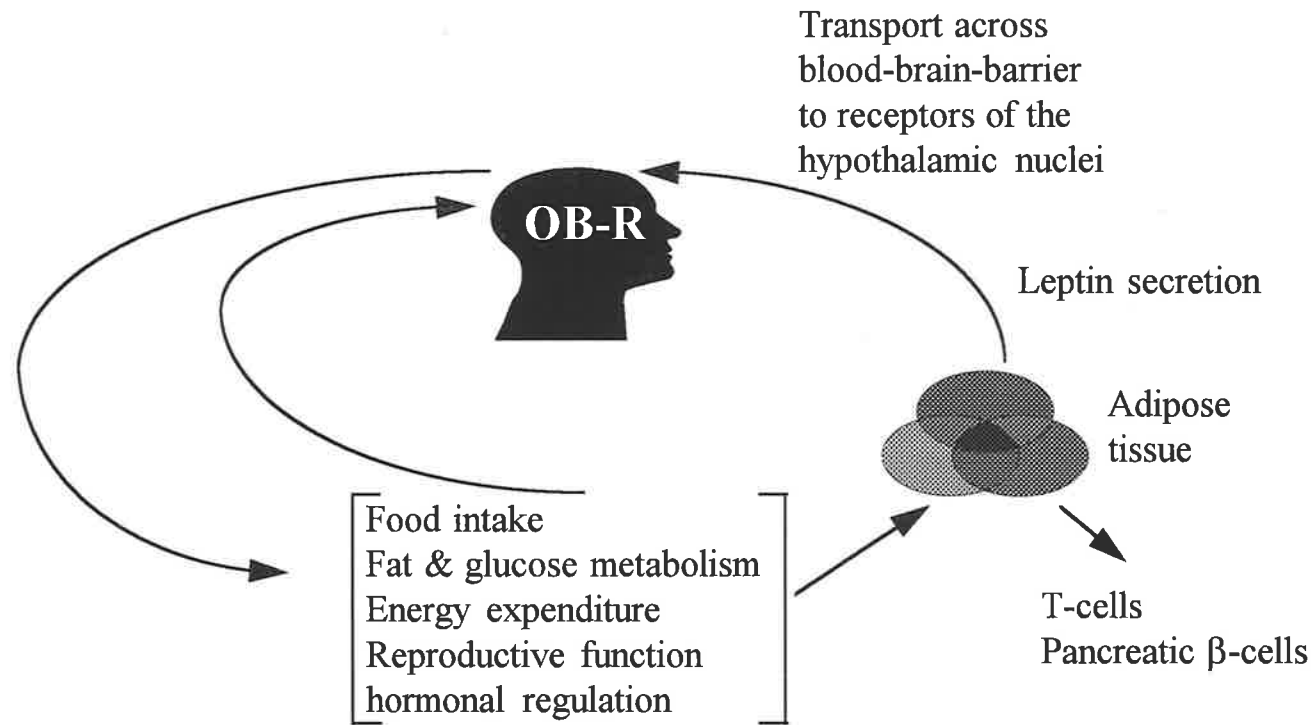


**Figure 1.2:** A schematic representation of several important elements of the leptin signalling pathway regulating energy balance. Leptin is secreted by adipocytes either as a 16K protein or bound to a soluble form of its receptor. Leptin crosses the blood brain barrier via a saturable transport system, where it acts on receptors in the hypothalamus (OB-R). The hypothalamus in turn regulates food intake, fat and glucose metabolism, energy expenditure, reproductive function and hormonal regulation. Leptin also has peripheral effects on T- cells, pancreatic islets and other tissues.

metabolic abnormalities of the *ob/ob* mouse (Campfield *et al* 1995, Halaas *et al* 1995, Pellemounter *et al* 1995). For example, peripheral leptin administration (10 mg/kg per day for 3 weeks) increases oxygen consumption, body temperature, and daily activity, and normalises blood glucose, and insulin levels of *ob/ob* mice (Pellemounter *et al* 1995).

In contrast to *ob/ob* mice, *db/db* mice have a mutation of the leptin receptor, and are consequently resistant to the action of leptin (Chen H *et al* 1996, Lee *et al* 1996). Plasma leptin levels are elevated in diabetic *db/db* mice, and leptin administration does not affect food intake or body weight (Campfield *et al* 1997, Halaas *et al* 1995).

There appears to be a range of sensitivities to the effects of leptin on body weight and food intake. Leptin deficient *ob/ob* mice are extremely sensitive to the actions of leptin, lean mice are less sensitive, and mice with diet-induced obesity are least sensitive. Lean rats and mice, and rats and mice with diet-induced obesity, respond to leptin administration in a similar fashion to the *ob/ob* mouse, but are less sensitive to its anorectic actions. Intravenous administration of leptin (30 µg) to lean rats decreases subsequent 7 hour feeding and body weight gain. Similarly, intracerebroventricular administration of leptin (3.5 µg) reduces food intake and body weight (Pellemounter *et al* 1995). However, unlike *ob/ob* mice, IP leptin administration (10 mg/kg per day for 3 weeks) has no effect on oxygen consumption, body temperature, activity levels, or plasma glucose and insulin levels in lean mice (Pellemounter *et al* 1995). Diet-induced obese mice show a reduction in food intake and body weight within 3 days after IP leptin administration (2 x 15 µg/day).

The injection of leptin (at doses much smaller than those which are effective peripherally) into the lateral or third ventricle of the hypothalamus reduces food intake and causes weight loss (Campfield *et al* 1996), suggesting that the hypothalamus is a major site of the action of leptin in mice. It has subsequently been shown that leptin acts directly on receptors within the central nervous system (Campfield *et al* 1995, Stephens *et al* 1995). The leptin receptor is discussed in section 1.5.2. Banks *et al* (1996) demonstrated in lean mice that approximately 75% of peripherally administered leptin crosses the blood brain barrier, via a saturable system. Uptake of leptin occurs in the choroid plexus, arcuate nucleus of the hypothalamus and in the median eminence.

Leptin-induced weight loss is the result of not only a decrease in food intake, but also an increase in energy expenditure (Halaas *et al* 1995, Pelleymounter *et al* 1995). The mechanisms by which leptin increases energy expenditure are uncertain.  $\beta$ -adrenoreceptor agonists inhibit leptin expression in adipose tissue (Collins & Surwit 1996, Mantzoros *et al* 1996, Moinat *et al* 1995, Trayhurn *et al* 1995). In addition, Collins & Surwit (1996) demonstrated that the  $\beta_3$  adrenergic receptor agonist, CL 316,243, prevented the development of obesity in mice fed a high fat diet, strongly decreasing leptin mRNA expression in white adipose tissue (WAT). It is not known whether blockade of  $\beta$ -adrenergic receptors attenuates leptin induced weight loss.

An important component of the effects of leptin on energy expenditure, particularly in animals, may involve activation of brown adipose tissue (BAT, see section 1.7.3.1) (Collins & Surwit 1996); leptin increases sympathetic activity in BAT, the kidney, the



hind limb and the adrenal glands (Haynes *et al* 1997). Increased sympathetic activity of BAT, associated with increased levels of uncoupling protein-1 (UCP1) mRNA, has been demonstrated in *ob/ob* mice treated with leptin. However, there is no evidence that leptin treatment effects UCP1 in wild-type mice. In rats, leptin has been shown to increase UCP-2 and UCP-3 mRNA expression in white adipose tissue and BAT. Uncoupling proteins and BAT are further discussed in section 1.7.

### 1.5.2 *The leptin receptor*

The leptin receptor (OB-R) was cloned from mouse choroid plexus in 1995 (Tartaglia *et al* 1995). The OB-R is a large single membrane spanning receptor and it belongs to the class I cytokine receptor family (Tartaglia *et al* 1995). The OB-R is encoded by the *db* gene, and is expressed widely throughout the brain, as well as in several peripheral tissues (Chen H *et al* 1996, Lee *et al* 1996, Steiner 1996). Localisation of the *ob* receptor within the hypothalamus is of particular interest as hypothalamic nuclei are important sites for the control of both food intake and energy expenditure. It has been shown that a defect in the OB-R is responsible for the obesity phenotypes of the *fatty* and Koletsky rats (Chua Jr *et al* 1996, Phillips *et al* 1996, Takaya *et al* 1996).

The OB-R exists as multiple receptor forms; the long intracellular domain form, short intracellular domain forms, and a soluble form that lacks a transmembrane domain (Cioffi *et al* 1996, Chen H *et al* 1996, Lee *et al* 1996, Tartaglia *et al* 1995). The short receptor isoforms (OB-R<sub>S</sub>) are expressed abundantly in the choroid plexus, lung, kidney and other peripheral tissues (Ghilardi *et al* 1996, Lee *et al* 1996), and may be

involved in transport of leptin from the blood into the cerebrospinal fluid (Tartaglia 1997). The long receptor isoform (OB-R<sub>L</sub>) is expressed predominantly in the hypothalamus (Ghilardi *et al* 1996, Mercer *et al* 1996, Schwartz *et al* 1996b, Tartaglia *et al* 1995). OB-R<sub>L</sub> is thought to be the signalling form of the receptor, mediating the central effects of leptin via its intracellular signal-transducing capabilities (Friedman & Halaas 1998, Tritos & Mantzoros 1997).

### 1.5.3 *Interaction of leptin with other neurotransmitters involved in energy balance*

Leptin is one of a number of peptide hormones and neurotransmitters involved in the neural network regulating food intake. Figure 1.3 shows interactions leptin and a range of other neurotransmitters within various nuclei of the hypothalamus.

Neuropeptide Y (NPY), a neuromodulator that potently stimulates of feeding (see section 1.6.4.3), is also major mediator of the actions of the leptin. NPY is increased in the hypothalamus of the *ob/ob* mouse, and NPY knockout attenuates the obesity and other abnormal features of these mice (Erickson *et al* 1996, Stephens *et al* 1995). Leptin inhibits NPY gene expression and secretion, and attenuates the increase in feeding following administration of NPY (Rohner-Jeanrenaud *et al* 1996, Schwartz *et al* 1996a,b, Stephens *et al* 1995). Similarly, expression of melanin-concentrating hormone (MCH), a hormone found primarily within the hypothalamus which increases food intake in rats (see section 1.6.4.8), is increased in the *ob/ob* mouse.

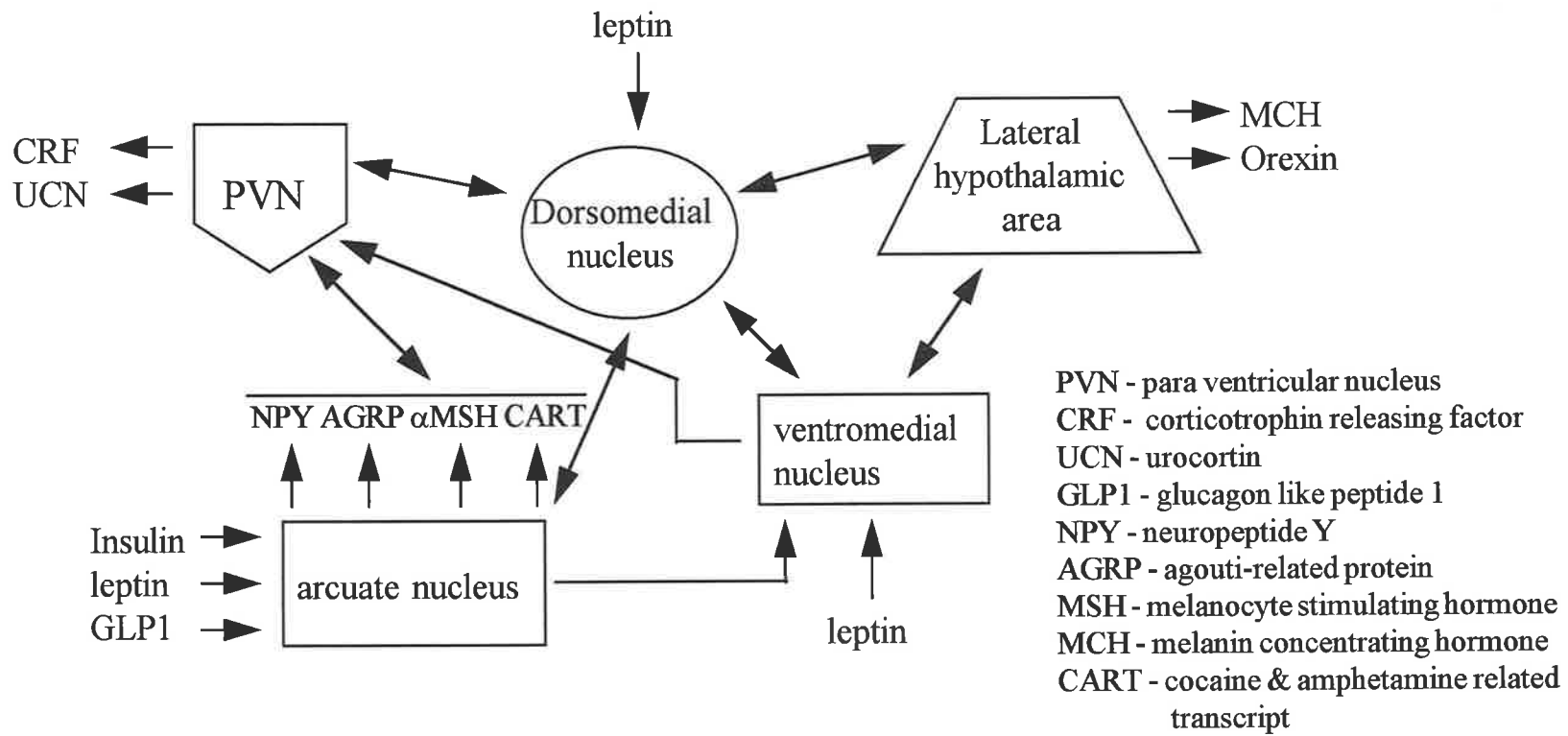


**Figure 1.3:** Schematic diagram of the interaction between leptin and other hypothalamic neurotransmitters

Cholecystokinin, a peptide released by the gastrointestinal tract, decreases food intake (see section 1.6.3.2) and potentiates the anorectic effects of leptin. Corticotrophin-releasing factor (CRF), a neuropeptide involved in the regulation of the hypothalamic pituitary adrenal (HPA) axis, decreases food intake in a range of species including mammals, fish and birds (see section 1.6.3.8). In rats, leptin administration increases levels of CRF mRNA in the paraventricular nucleus (PVN) of the hypothalamus, and stimulates the release of CRF from perfusion slices of the PVN. Pre-treatment with anti-CRF antibodies decreases the anorectic effect of single intracerebroventricular doses of leptin in rodents (Gardner *et al* 1998, Rohner-Jeanrenaud *et al* 1996, Schwartz *et al* 1996b). Cocaine and amphetamine regulated transcript (CART) is a hypothalamic peptide that decreases food intake (see section 1.6.3.12), and CART levels are increased in *ob/ob* mice (Kristensen *et al* 1998).

Melanocyte-stimulating hormone (MSH) is expressed in the hypothalamus of mammals and may be involved in the regulation of food intake (see section 1.6.3.11). MSH and its receptors are potential mediators of leptin action; leptin increases hypothalamic MSH (Friedman 1997). Abnormal melanocortin signalling in yellow agouti ( $A^Y$ ) or melanocortin-4-knockout mice induces obesity and leptin resistance (Huszar *et al* 1997, Halaas *et al* 1997) (see section 1.6.3.11). Pro-opio-melanocortin (POMC) is the pre-cursor of MSH and it is expressed on the same neurons as the OB-R. Leptin modulates POMC gene expression and agonists of MSH decrease the anorectic effect of leptin (Sato *et al* 1998).

A number of other neurotransmitters including glucocorticoids, bombesin, insulin, orexins, serotonin and noradrenaline are involved in the regulation of energy balance, downstream of the effects of leptin, within the nuclei of the hypothalamus, Fig 1.3.

#### 1.5.4 *Leptin resistance*

As leptin circulates in proportion to adipose tissue stores, obese animals (and humans) have higher levels of leptin than lean controls (except the *ob/ob* leptin deficient mouse), and leptin levels rise with increasing adiposity (Frederich *et al* 1995b, Maffei *et al* 1995). Obesity may therefore be associated with resistance to the actions of leptin.

The exact mechanism involved in leptin resistance remains uncertain, however numerous mechanisms may be involved. For example, studies in mice suggest that defects downstream of the leptin receptor in the hypothalamus may account for leptin resistance (Halaas *et al* 1997). The observation that in obese humans elevated plasma leptin is not associated with an increase in cerebrospinal fluid levels of leptin has led to the hypothesis that the leptin transport system may be defective or saturated in obese humans and animals (Caro *et al* 1996, Schwartz *et al* 1996a). In humans, it has been demonstrated that there is a threshold level (25-30 ng/ml) above which increases in serum levels are not translated into proportional increases in cerebrospinal fluid or brain leptin levels (*ie.* the transport system of leptin across the blood brain barrier is saturable). New Zealand obese mice are resistant to peripherally administered leptin but respond normally to centrally administered leptin, suggesting that impaired leptin transport into the CNS causes their obesity (Halaas *et al* 1997). In addition,

suggestions have been made that leptin has a limited ability to restrain the intake of highly palatable, high fat diets that are normally associated with an increase in adiposity (Widdowson *et al* 1997, and see chapter 7). The finding that C57BL/6 and AKR diet-induced obese mice have preserved sensitivity to centrally administered leptin, despite resistance to peripherally administered leptin, is consistent with these concepts (VanHeek *et al* 1997). Recently, a new family of cytokine inducible inhibitors of signalling have been identified. SOCS3 (suppressor of cytokine signalling-3) is a leptin inducible inhibitor of leptin signalling, and a potential mediator of leptin resistance in obesity (Bjorbaek *et al* 1998).

#### 1.5.5 *Leptin and the neuroendocrine response to starvation*

Although leptin has been traditionally viewed as an 'anti-obesity' hormone, increasing evidence suggests that it may actually play a role as an adaptive mechanism in an environment where food availability is limited. Decreased leptin concentrations may be the critical signal that initiates the neuroendocrine responses to starvation such as decreased thyroid hormone-induced thermogenesis, increased secretion of glucocorticoids and suppression of gonadal function (Ahima *et al* 1996, Legradi *et al* 1997, Tritos & Mantzoros 1997). Exogenous administration of leptin to starving mice blunts the neuroendocrine abnormalities associated with decreasing leptin levels due to food deprivation (*ie.* suppression of gonadal and thyroid axis, stimulation of adrenal axis) (Ahima *et al* 1996). These effects are mediated in part by NPY (Ahima *et al* 1996, Carro *et al* 1997, Yu *et al* 1997a). In humans, functional deficiency of leptin due to leptin receptor gene mutation result in abnormalities of the hypothalamic pituitary gonadal (HPG) and thyroid axes. Variations in serum leptin levels are related

to changes in adrenocorticotrophin hormone (ACTH) and cortisol levels in normal men, and to luteinizing hormone and oestradiol levels in normal women (Licinio *et al* 1997, 1998).

The *ob* gene coding sequence demonstrates a high levels of evolutionary conservation amongst vertebrate species, including humans and birds (see chapter 6), suggesting a conservation of leptins physiological functions. To date, however, there is no molecular information on leptin from a marsupial. The effect of exogenous leptin administration on fat mass, food intake and energy expenditure in marsupials has not been studied. In addition, it is not known whether an increase in fat mass is associated with decreased sensitivity to the actions of leptin in a marsupial.

## **1.6 THE REGULATION OF ENERGY INTAKE**

Eating starts (hunger) and stops (satiation) in a manner that is not clearly related to body weight, yet recognises caloric as well as specific nutrient requirements (Morley 1987). The regulation of food intake in mammals is a complex process that involves both central and peripheral regulatory mechanisms (Morley 1980). The hypothalamus is the part of the brain of primary importance in the regulation of food intake, acting as a transducer which integrates multiple sensory inputs and regulates nutritional homeostasis by activating or suppressing food seeking behaviour and energy expenditure (Morley & Levine 1983). The lateral hypothalamus is involved in the

onset of feeding, whereas the ventromedial hypothalamus mediates satiety in response to signals from the periphery (for review see Morley 1980). Peripheral signals to the brain arise from the gastrointestinal tract, as a result of plasma nutrient utilisation and the mass of adipose tissue ('the peripheral satiation system'). The hypothalamus receives inputs from other brain regions and coordinates the processes involved in energy intake ('the central feeding drive') (Morley 1980), Fig 1.4. It is now clear that the peripheral signals result from a complex interaction between gastrointestinal motility, hormone release and nutrient utilisation. within the hypothalamus, food intake is regulated by a complex neural network involving a large number of neurotransmitters, with inputs from and projections to many other areas of the brain.

#### 1.6.1 *The peripheral control of appetite*

The presence of food in the stomach leads to gastric distension, and subsequently to changes in motility of the proximal gastrointestinal tract, accompanied by the release of hormones (Smith 1983). Both gastric distension (mechanoreceptors) and the presence of nutrients in the proximal small intestine (chemoreceptors) appear to be important in the mediation of satiation. These effects are in turn mediated both by the release of gastrointestinal hormones, and by the activation of neural pathways via the vagus nerve (Morley 1990).



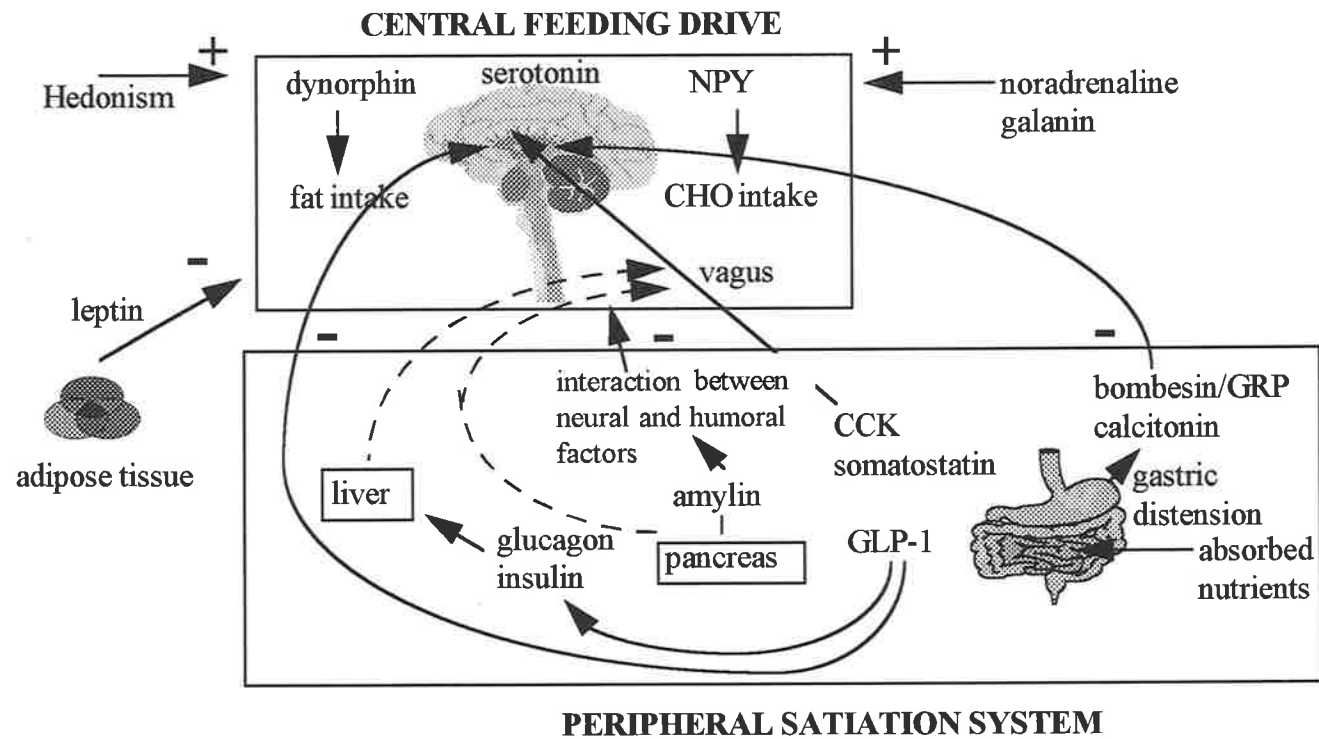


**Figure 1.4:** A model of mechanisms involved in appetite regulation. The ‘central feeding drive’ is responsible for the initiation of food intake, whereas the ‘peripheral satiation system’ is activated by the presence of food in the gastrointestinal tract.

Numerous studies in both animals and humans have demonstrated that gastric distension is a potent stimulus for satiation (Janowitz & Grossman 1949), and that this effect may be mediated by vagal mechanisms (Grundy *et al* 1994, Paintal 1954). For example, in sham fed dogs that have oesophageal fistulae, balloon distension of the stomach inhibits food intake (Janowitz & Grossman 1949), and in humans, gastric distension acutely reduces food intake by up to 30% in young adults (Geliebter 1988). The small intestine is also an important regulator of satiation, as a result of the slowing of gastric emptying and the release of hormones (Morley 1990). Hormones which are released in response to the presence of nutrients in the small intestine include cholecystokinin (CCK, see section 1.6.3.2), glucagon like peptide-1 (GLP-1, see section 1.6.3.4) and amylin (see section 1.6.3.1); all of which may modulate food intake (Morley 1990, Read *et al* 1994). There is evidence that gastric and small intestinal mechanisms may act synergistically to suppress food intake (Feinle *et al* 1996, Khan & Read 1992).

### **1.6.2      *The central control of appetite***

Two regulating centres of the hypothalamus which make up the central feeding drive have traditionally been described: i) the feeding centre located in the ventrolateral hypothalamus (VLH) appears to initiate feeding, whereas ii) the satiety centre located in the ventromedial hypothalamus (VMH) is responsible for producing the sensation of fullness (for reviews see Morley 1987, 1989, Morley & Gunion 1988). The satiety centre is associated with two major tracts of the central nervous system (CNS): i) the serotonergic pathway, which originates in the raphe nuclei of the pontine-midbrain area, and passes through the VMH (the raphe nuclei tract), and ii) the ventral

adrenergic bundle, which passes through the perifornical area in the vicinity of the VMH and projects into the lateral hypothalamus (Morley 1980). The VLH is associated with the dopaminergic nigrostriatal tract. These tracts also seem to be associated with the reward or pleasure centres of the brain (Morley & Levine 1983). The concept of the hypothalamus as having two distinct control centres is therefore an over-simplification. In fact, there is now known to be a complex neural network containing a number of hypothalamic nuclei, including the paraventricular, dorsomedial, ventromedial, and arcuate nuclei (Kalra *et al* 1999).

Electrical stimulation of the VLH (feeding centre) induces eating (Smith Jr 1961), as well as a series of anabolic responses, including increases in insulin release, hepatic glycogen synthesis and gastric acid secretion (Powley & Laughton 1981); damage to this area causes hypophagia and severe, fatal anorexia in otherwise healthy animals (Morley 1980), indicating that the satiety centre functions by inhibiting the feeding centre. Conversely, stimulation of the VMH (satiety centre) causes hungry animals to stop eating (Anand & Brobeck 1951), and also initiates a series of catabolic responses including activation of glycogenolysis, suppression of gastric acid secretion, and an increase in plasma levels of non-esterified fatty acid, glycerol and glucagon (Powley & Laughton 1981). Lesions in (or damage to) the VMH cause hyperphagia and, if food supply is abundant, result in hypothalamic obesity (Brobeck *et al* 1943). The feeding centre appears to be chronically active, and inhibited transiently by activity in the satiety centre following food ingestion. It is however, uncertain if the feeding and satiety centres simply control the desire to eat rather than food intake itself. For example, in rats with ventromedial lesions weight gain occurs for a while but food

intake then stabilises (Ganong 1985). After intake reaches a plateau, appetite mechanisms operate to maintain the new, higher weight. One theory to explain these observations is that the hypothalamic regulatory centres modulate the body weight 'set point', rather than food intake *per se* (Ganong 1985).

Signals from within the CNS and the gastrointestinal tract interact within the hypothalamus, which subsequently adjusts food intake, energy expenditure and many other pathways involved in the regulation of energy balance. Neurotransmitters within the CNS which may increase food intake include dopamine, endogenous opioid peptides, pancreatic polypeptide, catecholamines, orexins, galanin and melanin-concentrating hormone (Morley 1987, Read *et al* 1994). Neurotransmitters including serotonin, cholecystokinin, corticotrophin releasing factor and calcitonin have been demonstrated to inhibit food intake (Morley 1987, Read *et al* 1994). There is however, limited information about the physiological role of most of these neurotransmitters, in the absence of few specific antagonists for them. Possible neurotransmitters involved in the regulation of food intake are discussed below, and their effects on food intake are summarised in Table 1.1. The majority of these neurotransmitters have been tested in rodents, particularly in rats and mice. Many have also been demonstrated to have effects in humans. In contrast, there are relatively few studies that have determined the effects of these neurotransmitters on food intake in other species, and no studies have examined their effects on food intake in marsupials.

|                                                     | Effect on Food Intake |         |
|-----------------------------------------------------|-----------------------|---------|
|                                                     | Peripheral            | Central |
| <b>Pancreatic hormones</b>                          |                       |         |
| Insulin                                             | ↑ + ↓                 | ↓       |
| Pancreatic glucagon                                 | ↓                     | ↓       |
| Amylin                                              | ↓                     | ↓       |
| <b>Monoamines</b>                                   |                       |         |
| Dopamine                                            | ↑                     | ↑       |
| α-adrenergic regulators (eg. norepinephrine)        | ↓ or ↑                | ↑       |
| β-adrenergic regulators (eg. isoproterenol)         | ↓                     | ↓       |
| Serotonin                                           | ↓                     | ↓       |
| <b>Peptides released from the GI tract</b>          |                       |         |
| CCK (cholecystokinin)                               | ↓                     | ↓       |
| Bombesin                                            | ↓                     | ↓       |
| Gastrin releasing peptide                           | ↓                     | ↓       |
| Somatostatin                                        | ↓                     | ↓       |
| Motilin                                             | ↑                     | ↑       |
| GLP-1 (glucagon like peptide 1)                     | ↓ or no effect        | ↓       |
| <b>Other peptides and hormones</b>                  |                       |         |
| Pancreatic polypeptides                             |                       |         |
| eg. Neuropeptide y (NPY)                            | ↑                     | ↑       |
| Endogenous opioid peptides                          |                       |         |
| eg. β-endorphin/dynorphin                           | ↑                     | ↑       |
| Corticotrophin releasing factor (CRF)               | ↓                     | ↓       |
| Urocortin                                           | ↓                     | ↓       |
| Sauvagine                                           | ↓                     | ↓       |
| Calcitonin                                          | ↓                     | ↓       |
| Calcitonin gene related peptide                     | ↑ or no effect        | ↓       |
| Neurotensin                                         | ↓ or no effect        | ↓       |
| Galanin                                             | ↑                     | ↑       |
| Orexins                                             | ?                     | ↑       |
| MCH (melanin-concentrating hormone)                 | ?                     | ↑       |
| Melanocortin and agouti-related protein             | ?                     | ↓       |
| CART (cocaine and amphetamine-regulated transcript) | ↓                     | ↓       |
| TRH (Thyrotrophin-releasing hormone)                | ↓                     | ↓       |
| <b>Gonadal Hormones</b>                             |                       |         |
| Testosterone                                        | ↑                     | ↑       |
| Progesterone                                        | ↑                     | ↑       |
| Estrogen                                            | ↓                     | ↓       |
| <b>Other Neurotransmitters</b>                      |                       |         |
| GABA (γ-aminobutyric acid)                          | ?                     | ↑       |
| NMDA                                                | ↓                     | ↓       |
| NO                                                  | ↑                     | ↑       |
| <b>Signals from Adipose tissue</b>                  |                       |         |
| leptin                                              | ↓                     | ↓       |
| TNFα                                                | ↓                     | ↓       |

**Table 1.1:** Table summarising the effect of various neurotransmitters on food intake

### 1.6.3 *Neurotransmitters that may decrease food intake*

#### 1.6.3.1 *Pancreatic Hormones*

Insulin: Insulin is released from islet cells of the pancreas in response to hyperglycaemia. Centrally administered insulin and low doses of insulin administered peripherally by constant infusion decrease feeding in the rat, whereas insulin antibodies administered into the VMH enhance feeding (Strubbe & Mein 1977, VanDerWeele *et al* 1989). These studies suggest a suppressive effect of insulin on food intake. The major sensor for the acute effects of insulin on feeding is the liver. Fructose, which does not cross the blood brain barrier, blocks the effect of insulin on food intake, whereas ketones, which can be utilised as a fuel by the brain, do not affect insulin's actions on food intake (Friedman & Granneman 1983). In rats, the effects of insulin on food intake are not blocked by vagotomy, implying that the liver is not the only anatomical site at which insulin acts (Morley 1987). The effect of insulin on food intake remains controversial; some evidence suggests that insulin stimulates appetite (Morley 1990). In humans, acute peripheral insulin administration has been found to increase hunger and food intake in several studies, whereas other studies have found a suppressive effect (Barbour 1924, Morley 1987). There is no evidence, at least in humans, for a relationship between physiological levels of insulin and food intake or hunger, when blood glucose concentration is maintained at a constant level (Chapman *et al* 1998), and it has consequently been suggested that the ability of insulin to enhance feeding in several human studies is because it produces central glucoprivation (Bray 1985, Morley 1987).

Glucagon: Glucagon, a 29 amino acid pancreatic peptide released after consumption of a meal, has been shown to suppress feeding after peripheral administration in many species, including rats and humans via a vagally dependent pathway (Geary & Smith 1983, Penick & Hinkle Jr 1961). Peripheral administration of glucagon antibodies increases food intake in rats (Langhans *et al* 1982). Glucagon appears to act as a short-term regulator of satiety via pathways involving activation of gluco-sensitive cells in the liver; in long-term food deprived animals where liver glycogen stores have been depleted, the effects of glucagon are minimal, and intraportal glucagon infusions have more potent effects on feeding than systemic administration (Weick & Ritter 1986). In the rat, intracerebroventricular glucagon administration decreases food intake at lower doses than peripherally administered glucagon (Morley 1987).

Amylin: Amylin, a 37 amino acid peptide, is found predominantly in beta cells of the pancreas, the gastrointestinal tract (GIT) and the CNS of both rodents and humans (Guidobono 1998). Amylin has been demonstrated to decrease food intake in rats and mice when administered either centrally or peripherally (Morley & Flood 1991a, Morley *et al* 1997), and hepatic vagotomy does not prevent its anorectic effect (Lutz *et al* 1994, Morley *et al* 1994). The anorectic effect of peripheral amylin in rats can be blocked by the amylin receptor antagonist (CGRP-(8-37)) (Lutz *et al* 1994). In addition to its effects on food intake, amylin has been demonstrated to inhibit both gastric acid secretion and gastric emptying when administered either centrally or peripherally to rodents, an effect in part mediated by the CNS (Young 1997). Although the effect of amylin on food intake in humans has not been determined,

amylin levels increase in the peripheral circulation following a meal (Butler *et al* 1990).

#### 1.6.3.2 Cholecystokinin (CCK)

The prototypic satiation peptide CCK is a 33 amino acid polypeptide hormone, isolated from the GIT (porcine) in 1971 (Mutt & Jorpes 1971). In the gastrointestinal tract, CCK occurs in 2 forms (33 or 38 amino acids) (Bray 1992). The sulphated, N terminal, octapeptide form of CCK (CCK-8) contains the biological activity of CCK (Morley 1982), and is found in both the CNS and gut. CCK-8 decreases fat and CHO intake equally (Romsos *et al* 1987). CCK-A receptors are located primarily in the GI tract whereas CCK-B receptors are primarily located in the brain. The presence of fatty acids in the small intestine is the principal stimulus to CCK release, and CCK has multiple effects on the GIT, including contraction of the gallbladder, secretion of pancreatic juice rich in enzymes and inhibition of gastric emptying (for reviews see Morley 1987, 1990).

There is evidence that CCK release from the gut is involved in the physiological termination of a meal, and CCK has also been demonstrated to stimulate insulin and glucagon secretion. CCK decreases food intake in wolves, rats and a range of other species following both central and peripheral administration (for review see Morley *et al* 1985b). Although CCK is found in the CNS, the majority of studies indicate that it has mainly peripheral effects; CCK is more potent when given peripherally than centrally in most species (Morley *et al* 1995b). The CCK antagonist L-364, 718 reverses the ability of CCK to decrease food intake in rats and mice (Hewson *et al*



1988, Silver *et al* 1989). In humans, peripherally administered CCK induces dose dependent reductions in food intake, perhaps in part by slowing gastric emptying (Moran & McHugh 1982).

Exogenous (peripheral) CCK acts on CCK-A receptors of the vagus nerve, sending messages to the nucleus tractus solitarius within the medulla, and from there, to the PVN of the hypothalamus, resulting in a decrease in food intake or meal termination (Moran *et al* 1986). In rats, transection of the vagus nerve (which contains CCK receptors) inhibits the effect of CCK to increase satiety at low, but not high doses. CCK-8 has no effect on feeding in vagotomized humans (Morley 1987). Endogenous (central) CCK acts primarily on CCK-B receptors in the brain, and antagonists to the CCK-B receptor produce a greater increase in food intake than antagonists to CCK-A receptors (Dourish *et al* 1989). In addition, CCK has been demonstrated to increase activity of the sympathetic nervous system (SNS) when administered centrally, suggesting it may be involved in the modulation of energy expenditure (Bray 1992).

#### 1.6.3.3 *Bombesin (BBS) and gastrin releasing peptide (GRP)*

BBS, a 14 amino acid tetrapeptide was originally isolated from frog skin (Erspamer & Melchiorri 1975). GRP is the mammalian homologue of BBS, and is distributed widely in mammalian systems (Anastasi *et al* 1971). The highest concentrations of 'BBS-like' activity occur in the gut, particularly in the stomach. BBS like immunoreactivity and BBS binding sites are also present in the brain and lung (Moody & Pert 1979, Walsh & Holmquist 1976). BBS slows gastric emptying decreases food intake in rodents after both peripheral and central administration (Gibbs *et al* 1981,

Gibbs 1985, Morley & Levine 1981b). BBS has also been shown to decrease feeding in other mammals including wolves, baboons and humans (Levine *et al* 1984, McLaughlin & Baile 1981, Muurhainen *et al* 1983, Woods *et al* 1983). BBS has many actions that are similar to those of CCK on gut visceral functions (Bertaccini *et al* 1974, Hostetler *et al* 1989), and the effects of CCK and BBS to decrease food intake are additive, although BBS is less potent on a molar basis than CCK (Gibbs *et al* 1979). The exact mechanisms by which peripherally administered BBS mediates its effect on satiety are not fully understood. Subdiaphragmatic vagotomy fails to suppress the effects of BBS on food intake (Smith *et al* 1981b). In contrast, a combination of vagotomy and spinal visceral disconnection decreases the effects of bombesin on food intake (Stuckey *et al* 1985). BBS increases plasma concentrations of norepinephrine, suggesting a possible effect on energy expenditure (Bray 1992).

Gastrin releasing peptide (GRP) is a 27 amino acid peptide sharing a similar C-terminal decapeptide with BBS (McDonald *et al* 1979). GRP is released from the stomach in response to gastric distension. Peripherally administered GRP has been demonstrated to inhibit feeding in a range of species including rats, baboons and humans (Figlewicz *et al* 1985, Gutzwiller *et al* 1994, Stein & Woods 1982). As with BBS, histological studies have shown that GRP is present in various regions throughout both the CNS and gut of mammals (Yanaihara *et al* 1981). GRP has been shown to slow gastric emptying in rats (Yegen *et al* 1996). The physiological and behavioural actions of GRP remain to be determined.

#### 1.6.3.4 *Glucagon-like peptide-1*

In the gut, proglucagon is mainly processed to proglucagon 1-69 (glicentin) and two smaller peptides, glucagon-like-peptide-1 (GLP-1) and glucagon-like-peptide-2 (GLP-2) (Fehmann *et al* 1992). GLP-1 is present in at least two forms (1-37 and 7-37), and the truncated peptide is  $\alpha$ -amidated at the C-terminal end (Turton *et al* 1996). GLP-1 (7-36) amide is secreted by the intestine into the circulation after the ingestion of food and appears to be the main product of intestinal, but not pancreatic, proglucagon processing (Fehmann *et al* 1992). GLP-1 and its specific receptors are present in the hypothalamus; autoradiography of GLP-1 binding sites in the brain shows dense specific binding of GLP-1 in the hypothalamus, particularly the paraventricular nucleus (PVN), the central nucleus of the amygdala and the anterodorsal thalamic nucleus (Turton *et al* 1996).

GLP-1 (7-36) strongly stimulates insulin secretion, suppresses glucagon release, and inhibits gastric secretion (Fehmann *et al* 1992). GLP-1 stimulates insulin gene expression and insulin release via the adenylate cyclase/cAMP pathway; GLP-1 is thus involved at several levels in the regulation of insulin synthesis and secretion. There is evidence that GLP-1 is a short acting physiological mediator of satiety (Donahey *et al* 1998, Turton *et al* 1996); intracerebroventricular GLP-1 powerfully inhibits feeding in a dose dependent manner in fasted rats (Donahey *et al* 1998, Turton *et al* 1996), and in neonatal chickens (Furuse *et al* 1997). The sequence of GLP-1 is completely conserved in all mammalian species studied to date (Bell *et al* 1983, Drucker & Asa 1988, Heinrich *et al* 1984, Lopez *et al* 1983, Nishi & Steiner 1990, Orskov *et al*

1989, Seino *et al* 1986), and also conserved between mammals and birds (Hasegawa *et al* 1990), implying that it may play a critical physiological role.

The specific GLP-1 receptor antagonist exendin (9-39) blocks the inhibitory effect of GLP-1 on food intake in rats (Meeran *et al* 1999). Exendin alone has no influence on fast-induced feeding, but increases food intake in satiated rats and augments the response to the appetite stimulant NPY (Goldstone *et al* 1997, Turton *et al* 1996). There is no change in NPY mRNA expression following central GLP-1 injection (Turton *et al* 1996). Co-expression of leptin and GLP-1 mRNA in brain stem neurones, together with the observation that exendin blocks decreased food intake and body weight induced by leptin (Goldstone *et al* 1997), suggests that the GLP-1 pathway may be one of the mediators of the anorectic effects of leptin. However, GLP-1R knockout mice do not exhibit any abnormalities in feeding behaviour (Scrocchi *et al* 1996). Peripherally administered GLP-1 (up to 500 µg) does not affect early dark phase feeding in rats (Suzuki *et al* 1989).

Peripherally administered GLP-1 has been demonstrated to have powerful effects in humans. For example, in normal human subjects, peripherally administered GLP-1 decreases food intake, stimulates insulin secretion, delays gastric emptying and lowers blood glucose; in patients with non-insulin dependent diabetes (NIDDM) GLP-1 normalises both fasting and postprandial glycemia (for review see Drucker 1998).

#### 1.6.3.5 *Somatostatin*

The gastrointestinal hormone somatostatin is a cyclic tetradecapeptide that has been demonstrated to produce a vagally dependent inhibition of food intake after peripheral administration in baboons and rodents (Levine & Morley 1982). Somatostatin is found in both the CNS and gut, and is released from the gut in response to food intake, influencing a number of digestive processes. In rats, peripheral somatostatin acts synergistically with both CCK and glucagon to inhibit food intake, and it also inhibits the affect of bombesin on food intake (Morley 1987). In addition to its peripheral effects in rats, central administration of somatostatin reduces food intake, however this effect is possibly biphasic and depends on the animals fed state; somatostatin appears to be more effective at inhibiting food intake in fed, as compared to fasted, animals (Aponte *et al* 1984, Vijayan & McCann 1977). In humans, the effect of peripherally administered somatostatin remains to be fully determined, however studies to date have demonstrated no effect on food intake (Morley 1987).

#### 1.6.3.6 *Serotonin*

Hypothalamic injections of the monoamine serotonin, serotonin precursors, or fenfluramine (which induces serotonin release) decrease food intake in rodents (Morley 1980). Serotonin predominantly induces satiety rather than inhibiting hunger, as it reduces the size and duration of meals as well as the rate of eating, yet does not affect either the latency to meal onset, or meal frequency (Leibowitz 1986).

Serotonergic agonists reduce both fat and carbohydrate consumption (Orthen-Gambill & Kanarek 1982). There is evidence for an interaction between the serotonergic and

opioid feeding systems. For example, elevations of methionine-enkephalin and  $\beta$ -endorphin levels in the hypothalamus of fenfluramine-treated animals are not attenuated by treatment with D-amphetamine (Harsing Jr *et al* 1982, Majeed *et al* 1986). In contrast, others have failed to show effects of fenfluramine treatment on opiate agonist or antagonist feeding, suggesting that increased  $\beta$ -endorphin levels may represent a compensatory change in an attempt to overcome anorexia induced by serotonin (Morley 1987).

#### 1.6.3.7 $\beta$ -adrenergic control

$\beta$ -adrenergic receptors, located in the perifornical region of the lateral hypothalamus, induce reductions in food intake in response to norepinephrine (NE) and other  $\beta$ -agonists (Liebowitz 1980). Injection of isoproterenol (a  $\beta$ -adrenergic agonist) into the lateral hypothalamus of rats inhibits food intake, whereas  $\beta$ -adrenergic receptor blockers (eg. propranolol) stimulate food intake (Grossman 1962, Martin *et al* 1977). NE, which has both  $\beta$  and  $\alpha$  actions peripherally, stimulates feeding at low doses (the  $\alpha$  effect, see section 1.6.4.1), while higher doses suppress feeding (the  $\beta$  effect).

#### 1.6.3.8 Corticotrophin releasing factor, sauvagine and urocortin

CRF, a 41 amino acid peptide involved in the regulation of the hypothalamic pituitary adrenal (HPA) axis, is abundantly distributed throughout the brain and the GIT (Lovenberg *et al* 1995, Perrin *et al* 1995, Vaughan *et al* 1995). Both peripheral and central administration of CRF decrease food intake in a number of species. In addition to decreasing food intake, CRF inhibits gastric emptying and decreases luteinizing hormone secretion when administered peripherally to all species tested (for review see

Kalra *et al* 1999). Centrally administered CRF decreases food intake in fish, birds and mammals (DePedro *et al* 1997, Drescher *et al* 1994, Glowa *et al* 1992, Parrot 1990). Central CRF also induces a range of stress-like responses such as increased catecholamines, glucose, vasopressin, arterial blood pressure, heart rate and oxygen consumption (Drescher *et al* 1994, Emeric 1986, Morley 1987).

Two types of CRF receptor have been characterised (Chen *et al* 1993, Grigoriadis *et al* 1996, Perrin *et al* 1995); the CRF1 receptor is found predominantly in the pituitary and brain, while the CRF2 receptor is expressed in brain and peripheral tissues. There is evidence that the adrenocorticotrophin hormone (ACTH) releasing effect of CRF is mediated by the CRF1 receptor, while the appetite suppressing effects of CRF are mediated by the CRF2 receptor (Smagin *et al* 1998, Smith *et al* 1998, and see chapter 10). CRF appears to be involved in the complex neural network involved in appetite regulation. CRF inhibits norepinephrine, insulin, neuropeptide Y and dynorphin-stimulated feeding, whereas GABA ( $\gamma$ -aminobutyric acid) and serotonin inhibit CRF release (see Morley 1987).

Urocortin (UCN), a 40 amino acid peptide and an endogenous ligand for the CRF2 receptor, is expressed abundantly in the gastrointestinal tract (Vaughan *et al* 1995). As with CRF, UCN decreases food intake in rodents after both central and peripheral administration. UCN is however a more potent inhibitor of food intake in rats when compared to CRF when administered both centrally and peripherally (Spina *et al* 1996, Vaughan *et al* 1995).

Sauvagine is a 40 amino acid frog skin peptide with structural similarities to CRF (Gosnell *et al* 1983). Both central and peripherally administered sauvagine decrease food intake in rats; the effect of peripheral sauvagine is not blocked by vagotomy (Broccardo & Improta 1990, Gosnell *et al* 1983, Negri *et al* 1985). In addition, sauvagine has been demonstrated to have a longer lasting suppressive effect than CRF when given centrally to rodents (Negri *et al* 1985).

#### 1.6.3.9 *Calcitonin and calcitonin gene related peptide*

Calcitonin and calcitonin gene related peptide (CGRP) decrease food intake in rats after either central or peripheral administration, with the effects of central calcitonin lasting for up to 32 hours (for review see Morley *et al* 1985a). Although peripheral calcitonin decreases food intake, it is more potent when administered centrally, suggesting that the effects of calcitonin are due to a direct action on the CNS (Levine & Morley 1981a). Calcitonin binding sites are present in the CNS, primarily in the PVN of the hypothalamus (VanHouten *et al* 1982). The mechanism of calcitonin induced food suppression may be related to alterations in calcium flux by the neuronal tissue; both peripheral and central calcium administration increase feeding in some species, and this effect can be inhibited by calcitonin (Levine & Morley 1981a).

CGRP is a 37 amino acid peptide, expressed by the calcitonin gene in the CNS. CGRP is distributed in areas of the CNS classically associated with feeding. Either central or peripheral CGRP administration decreases food intake and gastric acid secretion in rodents (Krahn *et al* 1984, Lenz *et al* 1984), but the effects are less potent when CGRP is administered peripherally. The effects of CGRP are dependent on an intact



vagus (Tache *et al* 1984). CGRP has also been demonstrated to increase noradrenergic sympathetic outflow (Bray 1992). In rats, CGRP is a less potent inhibitor of food intake than calcitonin (Rosenfeld *et al* 1983).

#### 1.6.3.10 *Neurotensin*

Neurotensin, a 13 amino acid tridecapeptide originally isolated from bovine hypothalamus, has also been found in gut neurons. In rats, neurotensin decreases feeding when administered either peripherally or centrally (Luttinger *et al* 1982, Sandoval & Kulkosky 1992), and has also been shown to inhibit food intake induced by central norepinephrine administration. There is evidence that neurotensin and dopamine act together within the CNS to inhibit feeding (Hawkins *et al* 1986).

#### 1.6.3.11 *$\alpha$ -melanocyte stimulating hormone and Agouti related protein*

$\alpha$ -melanocyte stimulating hormone ( $\alpha$ MSH) is a peptide encoded for by the pro-opiomelanocortin (POMC) gene, which is also the pre-cursor of melanocortins such as ACTH and  $\beta$ -endorphin.  $\alpha$ MSH is expressed within the hypothalamus of mammals and inhibits food intake in rats following peripheral administration (see Kalra *et al* 1999).  $\alpha$ MSH also inhibits the increase in food intake observed in response to melanin-concentrating hormone (MCH) and CRF, but not neuropeptide Y (NPY) (Kalra *et al* 1999, Oohara *et al* 1993). The actions of  $\alpha$ MSH are probably mediated through the melanocortin-4 (MC4) receptor (see Kalra *et al* 1999). Central administration of MC4 receptor agonists inhibit feeding, and this effect can be inhibited by prior administration of melanocortin antagonists (Huszar *et al* 1997).

Mutation of the MC4 receptor in rats results in obesity, hyperphagia, hyperinsulinaemia and hyperglycaemia (Huszar *et al* 1997).

Agouti-related protein (AGRP), an endogenous antagonist of the melanocortin-4 receptor, is found within the hypothalamus. AGRP is structurally related to agouti protein, and over expression of either of these proteins results in obesity (Rossi *et al* 1998). For example, the agouti lethal ( $A^Y/a$ ) mouse is obese due to abnormal melanocortin signalling caused by the agouti protein acting on MC4 receptors (for review see Kalra *et al* 1999). Central administration of AGRP increases food intake in rats, and inhibits the action of  $\alpha$ -MSH (Rossi *et al* 1998). In rats, leptin induced inhibition of food intake and reduction in body weight are attenuated by co-injection of AGRP. AGRP mRNA expression is up regulated in *ob/ob* mice, and central leptin administration increases AGRP mRNA levels (Ebihara *et al* 1999).

#### 1.6.3.12 *Cocaine and amphetamine regulated transcript*

Cocaine and amphetamine regulated transcript (CART) are peptides found primarily in areas of the hypothalamus involved in feeding. A number of studies have suggested that CART may be a physiologically relevant anorectic signal. CART inhibits food intake in a dose-dependent manner when administered centrally to rats (Lambert *et al* 1998) and central administration of CART antibodies increase food intake (Kristensen *et al* 1998). As discussed in section 1.5.3, CART is regulated by leptin signalling.

### 1.6.3.13 *Thyrotrophin releasing hormone*

Thyrotrophin releasing hormone (TRH) is a tripeptide found throughout the CNS and the GI tract. Peripheral administration of TRH decreases food intake in rodents via a vagally dependent pathway, and central administration of TRH decreases feeding in rats (Morley & Levine 1980). The effects of TRH may be mediated by the cyclic dipeptide cHis-Pro (for reviews see Morley 1987, 1980). TRH also decreases feeding in hypophysectomized rats, suggesting that its effect is independent of the release of either thyrotropin from the pituitary, or thyroid hormones (Morley & Levine 1980).

## 1.6.4 *Neurotransmitters that may increase food intake*

### 1.6.4.1 *Catecholamines*

Studies in both animals and humans have established that the  $\alpha$ -adrenergic system is involved in the modulation of size, rather than frequency of meals, and that activation of this system causes preferential ingestion of carbohydrate rich foods (Leibowitz *et al* 1985, Shor-Posner *et al* 1985). When injected into the paraventricular nucleus (PVN) of the hypothalamus, norepinephrine (NE), the first amine to be implicated in appetite regulation, increases feeding through an  $\alpha_2$ -adrenergic effect (Goldman *et al* 1985). NE is thought to increase appetite by inhibition of the VMH satiety centre (Leibowitz 1980); lesions of the PVN attenuate this response. Depletion of NE with dopamine  $\beta$ -hydroxylase inhibitors (eg. FLA-63) decreases food intake, and activation of  $\alpha$ -adrenergic receptors with clonidine causes over eating (Anand & Brobeck 1951, Broekkamp & VanRossum 1972). There is evidence that  $\alpha_2$ -noradrenergic receptors stimulate feeding within the PVN by inhibiting the release of a feeding inhibitor, probably CRF. Adrenalectomy increases CRF in the PVN of rats, and attenuates the

NE feeding response, whilst replacement of corticosterone in these rats restores the ability of NE to stimulate feeding (Bhakhavtasalam & Leibowitz 1986).

#### 1.6.4.2 Dopamine

Although dopamine depletion induces hypophagia and dopamine agonists increase food ingestion, the role of dopamine in feeding behaviour is unclear (for review see Morley 1987). When administered centrally to rodents, dopamine increases feeding. A high fat, high calorie diet increases both dopamine and norepinephrine turnover. Chronic obesity is associated with decreased dopamine turnover in the dorsomedial nucleus of the hypothalamus in humans (Levin *et al* 1986). It has been suggested that dopamine interacts with the opioid feeding system to increase food intake; opiate antagonists inhibit dopamine-induced feeding, whereas dopamine antagonists inhibit opioid-induced feeding (Wood 1983).

#### 1.6.4.3 Neuropeptide Y (NPY)

NPY, a 36 amino acid peptide isolated from porcine brain, is one of the most abundant and potent stimuli for food intake identified within the CNS (for reviews see Kalra *et al* 1999, Morley 1987). NPY is released from the arcuate nucleus and its effects on appetite are mediated by the perifornical area, the VMN and PVN of the hypothalamus, and also structures associated with the fourth ventricle (Steinman *et al* 1994). NPY release is associated with most situations that require a feeding drive, such as fasting and hypoglycaemia. In contrast, absorption of nutrients inhibits NPY secretion coinciding with the termination of food intake (Kalra *et al* 1999). In rats, mice, pigs and ground squirrels, NPY injection into the PVN and VMH enhances

feeding (see Morley 1987). In rats, NPY predominantly drives CHO intake, particularly at the onset of the dark phase (Morley *et al* 1987, Sawchenko *et al* 1985). Chronic NPY administration to both lean and genetically obese animals induces hyperphagia and weight gain, and is associated with distension of the gastrointestinal tract. In addition, NPY has been found to reverse the weight loss associated with lateral hypothalamic lesions (Sahu *et al* 1988).

Although it is co-localised with norepinephrine, the effects of NPY are not directly related to the NE feeding system; the effects of NPY on food intake are not blocked by the dopamine antagonist phentolamine (Leibowitz 1989, Levine & Morley 1984). Insulin is closely associated with changes in NPY secretion. Central insulin decreases NPY mRNA in the arcuate nucleus, whereas insulinopenia increases it (see Kalra *et al* 1999).

Despite its ability to increase food intake in a range of species, NPY is only one of many factors responsible for the control of energy intake. NPY knockout mice do not show alterations in feeding behaviour (Erickson *et al* 1996), however when the knockout is produced in *ob/ob* mice, centrally administered NPY stimulates food intake. NPY is therefore likely to interact with other regulatory peptides and hormones such as leptin (the interaction of leptin with NPY is discussed in section 1.5.3). NPY appears to stimulate feeding through two separate receptor systems, Y1 and Y5 (Kalra *et al* 1999). NPY has been demonstrated to decrease sympathetic activity when injected into the PVN and third ventricle (Billington *et al* 1991).

#### 1.6.4.4 *Endogenous opioid peptides*

The endogenous opioid peptides (EOP's) are neurotransmitters that have been implicated in the reward or affective aspects of feeding, and have been shown to influence both food intake and food choice in a wide range of species ranging from humans to slugs (for review see Morley 1995). In addition to driving food intake, opioids are also involved in modulating locomotion, increasing analgesia, decreasing sexual interest, inhibiting the release of luteinizing hormone, and enhancing immune function (all processes pivotal in allowing species to focus on food intake). Opiates can effect food intake at both peripheral and central sites, however they are particularly effective in enhancing feeding when injected into the PVN and VMN of the hypothalamus (Gosnell *et al* 1986, King *et al* 1979). The DMN of the hypothalamus is also an important site of opioid action. In rats, exogenous and endogenous opioids (eg. morphine), stimulate food intake and increase fat consumption (see Morley 1987), and the opioid antagonist naloxone decreases food intake (particularly the intake of fat).

Both pharmacological and physiological evidence supports a major role for the kappa ( $\kappa$ ) -opioid receptor in maintenance of food intake; a wide range of  $\kappa$ -agonists increase feeding. For example, dynorphin is an endogenous  $\kappa$  ligand that has potent stimulatory effects on feeding (Morley & Levine 1983), via actions on the PVN of the hypothalamus. Interestingly, the predominant opiate receptor within the PVN is the  $\kappa$  receptor (Gosnell *et al* 1986, Mansour *et al* 1988). Central administration of antibodies to dynorphin decrease food intake (Carr *et al* 1987). Despite the potent effect of  $\kappa$  opioids on food intake, there is also evidence for delta ( $\delta$ ) and mu ( $\mu$ )

opioid receptor mediated effects. Peptide analogs with selective  $\delta$  activity enhance feeding after central administration, and  $\delta$  opioid antagonists decrease feeding.  $\mu$  opioid receptor agonists need to be administered at higher concentrations than  $\kappa$  and  $\delta$  agonists to promote food intake, and some  $\mu$  agonists, such as morphiceptin, have no effect on food intake. This suggests that  $\mu$  opioid receptors may be less important than  $\delta$  or  $\kappa$  in modulating food intake (Morley 1987). There is, however, evidence that the effects of opioids on fat intake may be regulated via the  $\mu$  opioid receptor. In invertebrates, the  $\mu$  opioid system drives foraging behaviour (Kavaliers & Hirst 1986). Injection of  $\beta$ -endorphin into the third ventricle reduces sympathetic activity of nerves supplying brown adipose tissue (Egawa *et al* 1993); opiates may accordingly be involved in the regulation of energy expenditure, as well as food intake.

#### 1.6.4.5 *Motilin*

Motilin, a 22 amino acid peptide, is expressed throughout the gastrointestinal tract, and is associated with inter-digestive myoelectrical complexes. Plasma motilin levels increase with fasting, and in both rodents and humans, peripherally administered motilin increases food intake (Christofides *et al* 1979, 1981, Garthwaite 1985). In fasted rats, the increased food intake observed in response to peripheral motilin is blocked by the opioid antagonists naloxone and naltrexone, suggesting an interaction between motilin and the opioid feeding system (Garthwaite 1985). Centrally administered motilin increases food intake for up to 2 hours in mice, and this effect is attenuated by the motilin receptor antagonist GM-109 (Asakawa *et al* 1998). The physiological function of motilin is yet to be fully determined, however, since motilin

receptors are present in areas of the brain specifically involved in food intake (Yano & Seino 1990), it is likely that motilin is involved in appetite regulation.

#### 1.6.4.6 *The Orexins*

Orexins were originally described as hypocretins because of their location within the hypothalamus and high sequence homology to the gut hormone secretin (DeLecea *et al* 1998). These peptides (named orexin-A and orexin-B) are found in neuronal cell bodies of the dorsal and lateral hypothalamus, and of the brain stem; the locus coeruleus of the hypothalamus is the region with the densest innervation of orexin neurones (DeLecea *et al* 1998, Nambu *et al* 1999). The orexin receptors, OX1 and OX2 are found exclusively in the brain, and have some similarity to receptors for other neuropeptides such as NPY. The expression of the genetic pre-cursor of both peptides (prepro-orexin) is up regulated in rats following a 48 hour fast (Sakurai *et al* 1998). Central administration of Orexin A increases food intake in rats, however this responsiveness is subject to circadian variation (Haynes *et al* 1999). Orexin B only weakly stimulates feeding in rats after central administration (Edwards *et al* 1999). These observations imply that orexins may be mediators in the central feedback mechanisms that regulate feeding. However, both peptides only marginally increase feeding and it is still questionable as to whether they play a major physiological role in appetite regulation. It may be that orexins are involved in satiety induced sleepiness; application of orexin A to the locus coeruleus increases both arousal and locomotor activity (Hagan *et al* 1999), and orexin knockout mice have narcolepsy (Sakurai *et al* 1999). Neurones containing orexin are located in segments of the spinal chord implicated in the modulation of autonomic activity (Ida *et al* 1999).



#### 1.6.4.7 *Galanin*

Galanin, a 29 amino acid neuropeptide originally isolated from the gastrointestinal tract, is found at high concentrations throughout the brain, with binding sites in a range of areas, but particularly in the PVN (Tatemoto *et al* 1983, Skofitsch & Jacobowitz 1985). Galanin increases food intake in rats when injected into the PVN, but not other hypothalamic sites (Kyrkouli *et al* 1986). Galanin coexists with noradrenaline in the PVN and increases feeding in part by releasing norepinephrine. This is supported by evidence that  $\alpha 2$  adrenergic receptor blocking drugs block or attenuate the effects of galanin (see Morley 1989). In rats, galanin increases fat and carbohydrate ingestion at the onset of the dark phase (Tempel *et al* 1988), suggesting a role for galanin in the modulation of the circadian feeding rhythm.

#### 1.6.4.8 *Melanin concentrating hormone*

Melanin concentrating hormone (MCH) is a 19 amino acid cyclic nonadecapeptide, found predominantly in the hypothalamus (for review see Kalra *et al* 1999). MCH has been shown to acutely increase feeding in rodents when administered centrally (Presse *et al* 1996, Rossi *et al* 1997), and also lowers plasma glucocorticoid levels. Mice lacking MCH are lean (50% less body fat than control mice at 17 weeks), have reduced food intake (ingested 12% fewer calories), and an increased metabolic rate, although activity is not increased (Shimada *et al* 1998). MCH is up regulated in genetically obese mice and fasted animals, and is down regulated by leptin (Kalra *et al* 1999, Qu *et al* 1996); hypothalamic MCH mRNA expression is decreased by leptin, and leptin prevents the increased food intake observed in response to centrally

administered MCH in rats (Sahu 1998). MCH thus appears to be a physiological stimulator of feeding behaviour.

#### 1.6.5 *Gonadal steroids and the regulation of food intake*

Various gonadal hormones have been implicated in the modulation of food intake and body weight (see Table 1.1), and many of these appear to interact directly with other neurotransmitters (for review see Morley 1987). Testosterone, for example, has been shown to increase food intake, with adult male rats consuming more than females; this difference is reduced by castration (Mooradian *et al* 1987). Oestradiol decreases food intake in rats, and there appears to be an interaction between gonadal steroids and the opioid feeding system, with oestradiol treated rats being less sensitive to opioid antagonism than ovariectomised rats (Bray 1974, Morley *et al* 1984).

#### 1.6.6 *Other neurotransmitters*

A number of other neurotransmitters have been implicated as regulators of appetite. These include GABA ( $\gamma$ -amino butyric acid) (for review see Morley 1980), N-methyl-D-aspartate (NMDA) (Stanley *et al* 1996), and nitric oxide (NO) (Moncada 1992). NO is a short-lived gas which is produced endogenously by the action of the enzyme nitric oxide synthase (NOS) on the amino acid L-arginine. In mice, inhibition of NOS using NG-nitro-L-arginine (L-NO-arg) decreases food intake (Morley & Flood 1991b, 1992). Long term administration of the nitric oxide synthase inhibitor N-nitro-L-arginine methylester (L-NAME) decreases food intake and produces weight loss in genetically obese (*ob/ob*) mice (Morley & Flood 1994). The *ob/ob* mouse is far more sensitive to L-NAME than its genotype control the *ob/c* mouse, and has elevated

levels of NOS and its mRNA in the hypothalamus (Morley & Flood 1994). Recent studies have demonstrated that inhibition of central NOS activity, using peripherally administered L-NAME, decreases food intake in the marsupial *Sminthopsis crassicaudata*, and that this effect is blocked by L-arginine (L-Arg) (Vozzo *et al* 1999).

#### 1.6.7 *Signals arising from adipose tissue*

Signals arising from adipose tissue involved in the regulation of food intake include leptin (which is discussed in section 1.5) and tumour necrosis factor-alpha (TNF $\alpha$ ). TNF $\alpha$  is a multifactorial cytokine produced by adipose tissue, that has been demonstrated to modulate many aspects of adipocyte metabolism, including transcriptional regulation, glucose and fatty acid metabolism and hormone receptor signalling (for review see Sethi & Hotamisligil 1999). TNF $\alpha$  levels increase with increasing body fat mass. In rats, peripherally administered TNF $\alpha$  decreases food intake and body weight, and increases rectal temperature, resting oxygen consumption (VO $_2$ ), brown adipose tissue (BAT) thermogenic activity and muscle catabolism (Coombes *et al* 1987).  $\beta$ -adrenergic blockade with propranolol prevents the increase in VO $_2$  observed in response to TNF $\alpha$ , and activation of BAT is prevented by denervation of BAT (Coombes *et al* 1987). Anti-TNF $\alpha$  antibodies have been demonstrated to increase food intake in tumour bearing rats (Smith & Kluger 1993). Centrally administered TNF $\alpha$  has been demonstrated to suppress food intake in rats by acting directly on the CNS (Plata-Salam *et al* 1988).

Significant correlations exist between circulating leptin, TNF $\alpha$  and body fat stores in both rodents and humans. TNF $\alpha$  has been implicated in the regulation of leptin production; TNF $\alpha$  treatment increases leptin production *in vivo*, and obese mice lacking TNF $\alpha$  receptors have lower circulating leptin levels compared to their obese controls (Sethi & Hotamisligil 1999). However, obese TNF $\alpha$ -deficient mice still exhibit higher leptin levels than their lean counterparts (Kirchgessner *et al* 1997), and increased levels of leptin secreted in response to TNF $\alpha$  are not reflected by increased leptin mRNA levels in adipose tissue (Kirchgessner *et al* 1997), suggesting that TNF $\alpha$  is not a major regulator of leptin.

## 1.7 THE REGULATION OF ENERGY EXPENDITURE

Body weight is maintained by a balance between energy intake and energy expenditure. Total energy expenditure is determined by the sum of basal metabolic rate (BMR), physical activity and the generation of heat by metabolic fuels (thermogenesis) (Himms-Hagen 1989). Thermogenesis includes diet-induced thermogenesis (DIT), and shivering and non-shivering thermogenesis.

### 1.7.1 *Basal metabolic rate*

BMR is the energy expenditure of the body while at rest, during the post-absorptive phase (several hours after eating when all food has been digested and absorbed) (Dietz 1989). This represents the energy requirement for maintaining life; comprising predominantly maintenance of heart rate, breathing, nerve transmission,

electrochemical gradients across membrane cells, and the energy cost of protein turnover required to maintain cells. In humans, basal metabolic rate accounts for nearly 60%, the dietary thermogenic response about 10% and physical activity about 30% of total energy output (Weigle 1994). In mammals, including rodents and humans, BMR is higher in obese as compared to lean animals, increases with overfeeding and decreases with weight loss (Dietz 1989). The factors influencing BMR in marsupials are controversial and remain to be fully determined.

### 1.7.2 *Physical activity*

Physical activity is the most variable component of energy expenditure, and has a major influence on overall energy balance by affecting total energy expenditure, fat balance and food intake. The total amount of energy expended during activity depends on the characteristics of the physical activity (mode, intensity, duration and frequency), and of the individual performing the activity (body size, level of habituation). A reduction in physical activity is one of the potential mechanisms by which mammals can conserve energy.

### 1.7.3 *Non-shivering thermogenesis*

Non-shivering thermogenesis (NST) is the process whereby heat is generated from metabolic fuels in the absence of muscular contractions (Sellers *et al* 1954). More than 40 years ago it was established that the conversion of metabolic fuels to heat, in excess of the BMR, occurred as a result of noradrenaline (NA) release from sympathetic nerve terminals (see Himms-Hagen 1985). For example, in adult rats, subcutaneous injection of NA (0.25 mg/kg) produces a 10% increase in resting

oxygen consumption ( $\text{VO}_2$ ), and in human males administration of NA (0.42 mg/kg) induces a 6.6% increase in resting  $\text{VO}_2$  (Liu *et al* 1995, Rothwell *et al* 1982). NST is regulated by the activity of the sympathetic nervous system, and mediated by the actions of a protein, located on the inner mitochondrial membrane, known as uncoupling protein-1 (UCP1) (Bouillaud *et al* 1986, Jacobsson *et al* 1985). While homologous proteins (UCP2 and UCP3) have been identified, their role (if any) in thermogenesis has not been established (Boss *et al* 1997a,b, Fleury *et al* 1997, Gimeno *et al* 1997). Non-shivering thermogenesis is important in many mammals during arousal from hibernation or torpor, and for the maintenance of body temperature in the cold (Foster 1984, Himms-Hagen 1984, Jansky 1973).

#### 1.7.3.1 *Brown fat, UCP1 and the regulation of energy balance*

In 1963, brown adipose tissue (BAT) was identified as the thermogenic effector of arousal in hibernators (see Himms-Hagen 1985). BAT is the principal organ for the production of thermoregulatory heat by non-shivering mechanisms and diet-induced thermogenesis in many mammals (Foster 1984, Himms-Hagen 1984, Rothwell & Stock 1979a, 1983). Heat is generated intracellularly by controlled uncoupling of oxidative phosphorylation mediated by UCP1, which is found exclusively in BAT (Cannon & Nedergaard 1985, Nicholls & Locke 1984, Trayhurn 1993), Fig 1.5. In rodents, UCP1 expression and activity are increased in response to cold exposure, food intake and catecholamines (Hamman & Flier 1996, Himms-Hagen 1985, Klaus *et al* 1991, Ricquier *et al* 1991). Based on the detection of UCP1 expression, BAT has been identified in a large range of mammals (Trayhurn 1993). In some species, UCP1 is only evident for a brief period of post-natal life (Trayhurn 1996). In lambs, cattle,

goats and reindeer, UCP1 is found in adipose tissue deposits at birth, and the concentration of the protein falls rapidly during the early post-natal period (Casteilla *et al* 1987, Soppela *et al* 1991). In new born humans, UCP1 is thought to play a role in thermoregulation during cold exposure, but UCP1 appears to play only a minor role, if any, in the regulation of thermogenesis in adult humans, probably due to the low level of UCP1 mRNA expression (Garruti & Ricquier 1992, Lean & James 1983, Lean *et al* 1986). In other species, such as the domestic pig and birds, UCP1 appears to be absent (Saarela *et al* 1991, Trayhurn *et al* 1989). To date, the presence of BAT in marsupials is controversial as UCP1 has not been detected (see section 2.3.2.1). Apart from activity of the sympathetic nervous system and food intake, a number of other factors may influence the thermogenic activity of BAT, including photoperiod (Mauer & Bartness 1994, McElroy *et al* 1986), gonadal steroids (Wade *et al* 1985) and leptin (Trayhurn *et al* 1995).

UCP1 deficient mice consume less oxygen after treatment with  $\beta$ 3-adrenergic receptor agonists, and are cold sensitive implying that UCP1 is an important mediator of thermoregulation (Enerback *et al* 1997). BAT has also been implicated as an important site of facultative energy expenditure, and the absence of BAT in transgenic mice leads to obesity; the increase in fat mass occurring prior to any increase in food intake (Lowell *et al* 1993). This latter observation is consistent with the concept that regulation of energy expenditure may be uncoupled from food intake and primarily be responsible for alterations in body weight. In contrast, the UCP1 knockout mouse is not obese, suggesting that other factors in BAT are either more important than, or are able to compensate for the absence of UCP1 (Enerback *et al* 1997). Interestingly,

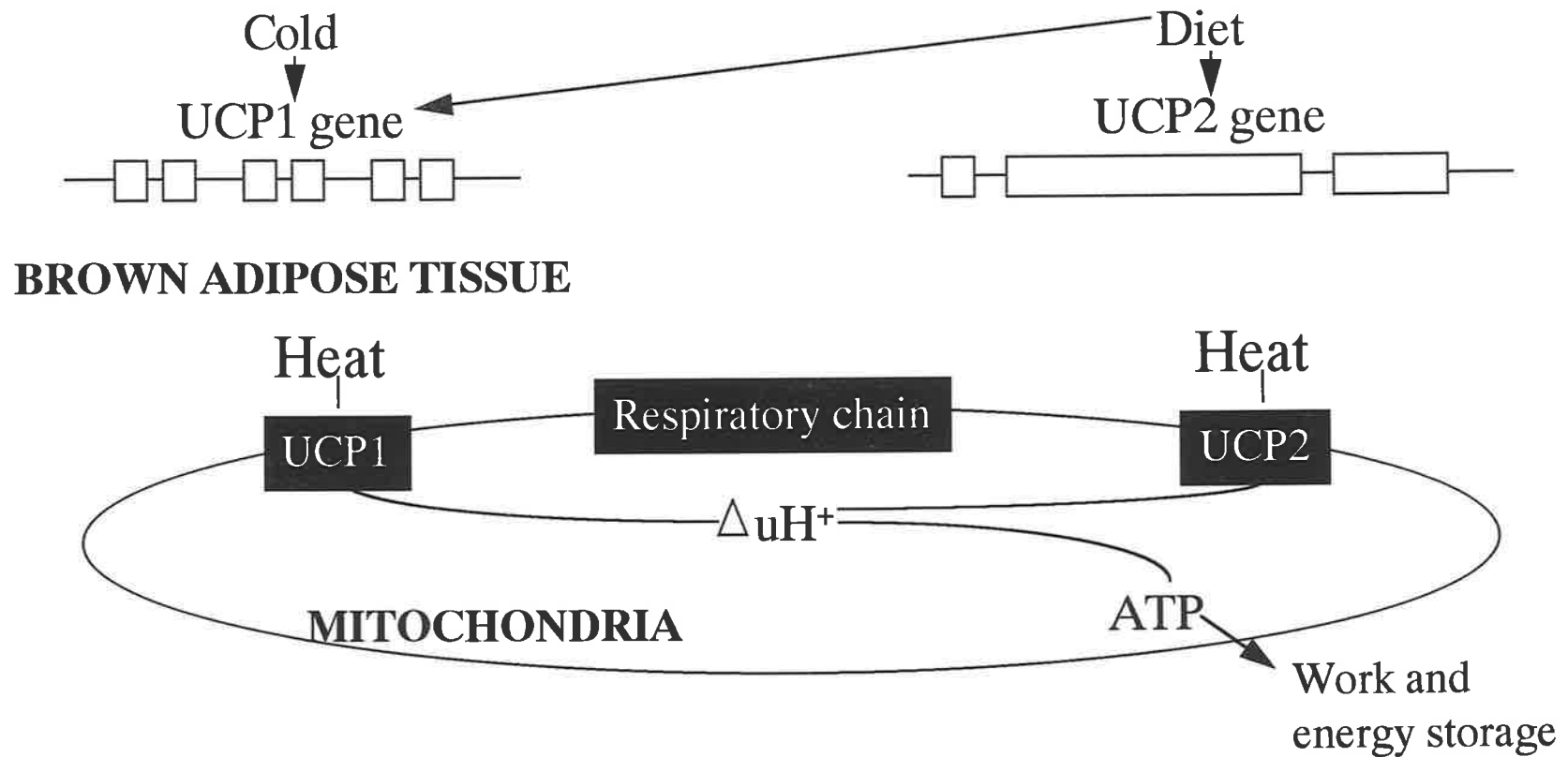


**Figure 1.5:** Uncoupling proteins-1 and -2 and thermogenesis. UCP1 is found exclusively in BAT, whereas UCP2 has a wide tissue distribution.



brown adipose tissue UCP2 mRNA levels (see section 1.7.3.3) are up regulated in UCP1 knockout mice by a magnitude of five fold, suggesting BAT UCP2 may compensate for defects in UCP1, thereby maintaining a stable body mass (Enerback *et al* 1997). Although the UCP1 knockout mouse remains cold sensitive, there is no evidence that the increase in UCP2 is associated with increased thermogenesis (Enerback *et al* 1997).

#### 1.7.3.2 *Adrenergic receptors and thermogenesis*

In BAT, thermogenesis is initiated by the release of NA from the sympathetic nervous system (Jansky 1973, Wunder 1979). In rodents, BAT and WAT contain both  $\alpha$ , and  $\beta$ 1, 2 and 3 adrenergic receptors (AR) (Arch & Kaumann 1993, Nedergaard *et al* 1996, Zaagsma & Nahorski 1990). The  $\beta$ 3 AR is the major AR found in the membranes of brown adipocytes, where it mediates thermogenesis, hypertrophy of BAT and lipolysis, an effect also seen with  $\beta$ 3 (as well as  $\beta$ 1 and 2) agonists in WAT (Susulic *et al* 1995). Transgenic mice, with a targeted disruption of the  $\beta$ 3 adrenergic receptor gene, become obese, confirming the importance of thermogenesis in the regulation of body weight (Susulic *et al* 1995). Further evidence for a role of  $\beta$ 1-AR in regulating energy balance comes from experiments using transgenic mice that over-express the  $\beta$ 1-AR in WAT and BAT, where stimulation of lipolysis, BAT proliferation and partial resistance to diet-induced obesity have been observed (Grujic *et al* 1997, Soloveva *et al* 1997). However, the physiological role of the  $\beta$ 3-AR (and the role of other AR's) in thermogenesis in other species is controversial. In particular, the role of adrenergic receptors in the regulation of thermogenesis in marsupials remains to be confirmed (see section 2.3.2.2).

### 1.7.3.3 *Uncoupling protein-2 (UCP2)*

UCP2 is a recently described mitochondrial protein which is 59% homologous to UCP1 at the amino acid level in humans (Fleury *et al* 1997). When expressed in yeast UCP2 has a greater effect on mitochondrial membrane potential than UCP1 (Fleury *et al* 1997, Gong *et al* 1997), implying that UCP2, like UCP1 can uncouple oxidative respiration within mitochondria, Fig 1.5. However, it is not known whether UCP2 is important in the regulation of thermogenesis *in vivo*. UCP2 is widely expressed in both rodent and human tissues including BAT, WAT, skeletal muscle, brain, spleen, heart and liver. (Fleury *et al* 1997, Solanes *et al* 1997). UCP2 is involved directly in nutrient partitioning, mediating lipid oxidation by transporting fatty acids out of the mitochondria for utilisation as a fuel source; an increase in free fatty acids increases the expression of UCP2 in muscle (Jezek *et al* 1997, Simoneau *et al* 1998, Solanes *et al* 1997). In rodents, cold exposure is associated with an increase in UCP2 mRNA expression in BAT, and cardiac and skeletal muscle (Boss *et al* 1997b, Gong *et al* 1997, Samec *et al* 1998, Vidal-Puig *et al* 1997). Feeding (particularly of fat) increases UCP2 expression in WAT (Hun *et al* 1999). Furthermore, in response to a high fat diet, the expression of UCP2 mRNA is increased more in an obesity resistant (A/J) strain of mouse than an obesity prone (C57B1/6J) strain of mouse (Fleury *et al* 1997). In both rodents and humans, fasting does not alter UCP2 mRNA expression in BAT, but does increase UCP2 mRNA expression in WAT and skeletal muscle (Brun *et al* 1999, Langin *et al* 1999, Samec *et al* 1999). This suggests a potential role for UCP2 in the metabolic adaptation to fasting and the partitioning of nutrients for utilisation or storage. It has been suggested that skeletal muscle UCP2 may mediate lipid oxidation by transporting fatty acid anions out of the mitochondria so that they can be utilised as

fuel (Simoneau *et al* 1998, Solanes *et al* 1997). Longer-term food restriction in humans decreases UCP2 mRNA expression in WAT (Millet *et al* 1997). It therefore appears that there is differential regulation of UCP2 in different tissues. For example, BAT and skeletal muscle UCP2 may regulate thermogenesis (Boss *et al* 1997b, Gong *et al* 1997, Samec *et al* 1998), while skeletal muscle UCP2 may be involved in the regulation of lipids as fuel substrates (Boss *et al* 1997b, Samec *et al* 1998). Although there is little evidence in humans to support a significant role for UCP2 in the regulation of thermogenesis, UCP2 expression is associated with RMR, and a polymorphism in exon 8 of the UCP2 gene is linked to percentage body fat (Oberkofler *et al* 1998). In obese humans, UCP2 mRNA expression is reduced in WAT compared to lean humans; this suggests that a defect in UCP2 expression may possibly be responsible for the impaired oxidation of lipids in obesity (Oberkofler *et al* 1998). UCP2 has yet to be identified in a marsupial.

#### 1.7.3.4 UCP3

UCP3, also a recently described mitochondrial protein, is 57% similar to UCP1 and 73% similar to UCP2 at the amino acid level in rats (Boss *et al* 1997a,b). Unlike the wide tissue distribution of UCP2, UCP3 is expressed primarily in skeletal muscle, and at low levels in both white and brown adipose tissue (Boss *et al* 1997a, Vidal-Puig 1997). The abundance of UCP3 in skeletal muscle of humans and other mammals, implies that it may function as a mediator of thermogenesis. However, there is no evidence that this is the case *in vivo* and, like UCP2, UCP3 expression is increased in response to elevated fatty acid levels during fasting (Millet *et al* 1997, Samec *et al* 1998, Weigle *et al* 1998). UCP3 may therefore also play an important role in

regulating energy balance via alterations in nutrient partitioning. Skeletal muscle UCP3 in particular, has been demonstrated to increase with fasting in both rodents and humans (Gong *et al* 1997, Langin *et al* 1999), and has been implicated in the regulation of lipids as fuel substrates. Interestingly, UCP3 from humans has two RNA transcripts (UCP3<sub>L</sub> and UCP3<sub>S</sub>), predicted to encode long (312 amino acids) and short (275 amino acids) forms (Solanes *et al* 1997). UCP3 mRNA levels in muscle correlate with percent total body fat in humans (Bao *et al* 1998). Muscle UCP3 levels are decreased in hypothyroid, and increased in hyperthyroid rats, suggesting that the responses to hormones may be a potential mechanism by which UCP3 effects thermogenesis (Gong *et al* 1997, Reitman *et al* 1999). However, since free fatty acids appear to regulate UCP3 expression and thyroid hormone stimulates lipolysis, thyroid hormone may not be involved directly in the regulation of UCP3 expression (Reitman *et al* 1999). Furthermore, the  $\beta$ 3-adrenergic agonist CL214613 increases WAT UCP3 levels, suggesting another possible pathway of UCP3 induced thermogenesis (Gong *et al* 1997). UCP3 has not been identified in any marsupial species.

#### 1.7.4 *Diet-induced thermogenesis*

The increase in energy expenditure caused by food intake is known as diet-induced thermogenesis (DIT), originally termed the thermic effect of feeding (Liverini *et al* 1995). DIT is defined as the postprandial (after eating) increment in energy expenditure above the resting metabolic rate and is generally expressed as a fraction of the energy content of the food consumed (Bjorntorp & Brodoff 1992). DIT comprises obligatory and facultative components (Himms-Hagen 1989, Liverini *et al* 1995). The obligatory component is the energy used to digest, transport and store

food (Himms-Hagen 1989). Macronutrient intake (particularly of carbohydrate) stimulates the sympathetic nervous system (SNS) and the resulting catecholamine-mediated increase in metabolic rate is known as the facultative component of DIT; the latter can be blocked by the  $\beta$ -adrenergic antagonist propranolol (Liverini *et al* 1995, Rothwell & Stock 1979a,b). Although DIT has been demonstrated to occur in a range of mammals including humans and rodents, DIT has not previously been demonstrated in a marsupial (see section 2.3.2.3).

DIT may account for up to 15% of total energy expenditure (Foster 1984). 'Cafeteria' fed rats over-eat and consequently have increased thermogenesis as a result of activation of BAT (Brooks *et al* 1980, Stock & Rothwell 1979, Tulp *et al* 1982). DIT occurs in other animals including humans but the mechanisms are poorly understood (Rothwell & Stock 1979a,b, 1982, Welle *et al* 1981, Zed & James 1986). In humans, there is an inverse relationship between fat mass and DIT; DIT appears to be important to enable mammals to resist weight gain by the dissipation of excess calories as heat (Landsberg & Young 1983, Zed & James 1986).

Stability of body composition is dependent on the capacity to oxidise a fuel mixture matching the nutrient mixture consumed, or to compensate for nutrient excess or deficiency. This allows adaptation to substantial variations in the macronutrient content and availability of food (Flatt 1987). The magnitude of DIT is dependent on both the nutritional status of the animal, as well as the macronutrient content of the food consumed (Landsberg & Young 1983, Macdonald & Webber 1993, Matsuo *et al* 1995, Yang *et al* 1990). Food deprivation decreases energy expenditure in humans

and rodents, at least in part by decreased activity of the sympathetic nervous system (SNS) (Landsberg & Young 1983, Macdonald & Webber 1993, Yang *et al* 1990). Moreover, hyperphagia has been shown to activate the SNS (Landsberg & Young 1983). Feeding different macronutrients of similar caloric value also affects oxygen consumption (Baba *et al* 1982, Bjorntorp & Brodoff 1992). For example, the ingestion of carbohydrate has been shown to elicit a greater thermogenic response than an equicaloric load of fat (Blaak & Saris 1996, Landsberg *et al* 1984). The difference in the magnitude of DIT between carbohydrate and fat has been attributed to alterations in the level of activation of the SNS; the oxidation of carbohydrates causes a greater increase in NA turnover compared with the oxidation of fat and protein (Blaak & Saris 1995, Landsberg *et al* 1984). High fat diets are also more likely to result in obesity, although rodent strains susceptible and resistant to diet-induced obesity have been described (Greenberg *et al* 1999, Levin & Keesey 1998, Surwit *et al* 1998).

#### 1.7.5 *Impact of thermogenesis on the regulation of body composition*

There is substantial evidence that thermogenesis plays a primary role in the regulation of body fat in mice. For example, i) the *ob/ob* mouse is obese secondary to leptin deficiency (Zhang *et al* 1994). Soon after birth, these mice manifest a decrease in thermogenesis due to a decrease in activity of BAT; the increase in body weight precedes an increase in food intake, ii) BAT knockout mice develop obesity, which precedes an increase in food intake (Lowell *et al* 1993), iii)  $\beta$ 3-AR knockout mice have increased fat stores but a similar food intake to wild type mice (Grujic *et al* 1997), iv) induction of UCP1 expression in WAT of C57BL6/J mice reduces dietary

obesity (Surwit *et al* 1998), v) mice with a knockout of the RII $\beta$  isoform of protein kinase A (PKA, expressed predominantly in BAT, WAT and brain), are healthy but have markedly diminished WAT, normal food intake, and are protected against diet-induced obesity (Cummings *et al* 1996). In BAT of these mice, there is a compensatory increase in the RI $\alpha$  isoform, which almost entirely replaces RIIb. As a result, UCP1 activity, metabolic rate and body temperature increase (Cummings *et al* 1996).

#### 1.7.6 *Conclusion*

This chapter has briefly reviewed the literature pertaining to the regulation of body fat stores, food intake and energy balance in mammals. In particular, it has demonstrated that, although there is much information available on the regulation of these processes in mammals, particularly rodents, there is very little known with regards to the regulation of fat mass, food intake and energy balance in marsupials. An examination of these processes in marsupials will provide new and important information on marsupial biology, from both a comparative physiological and an evolutionary perspective. These issues are further explored in chapter 2.

## CHAPTER 2

**RELEVANCE OF A MARSUPIAL MODEL FOR STUDIES  
RELATING TO THE REGULATION OF BODY FAT STORES,  
APPETITE, AND ENERGY EXPENDITURE****2.1 SUMMARY**

Although the regulation of body fat stores, food intake and energy balance has been studied in many mammals, including humans, there is very little information on these processes in marsupials. The vast majority of studies in marsupials have been ecological, reproductive, anatomical or genetic. Studies of the regulation of energy balance in marsupials are also of interest in an evolutionary context because of the early separation of the marsupial and eutherian lineages. This chapter reviews current knowledge of the regulation of food intake and energy balance in marsupials, and introduces the dasyurid marsupial *Sminthopsis crassicaudata*, the marsupial model used in the research presented in this thesis.



## 2.2 THE EVOLUTION AND TAXONOMY OF MARSUPIALS

The ancestral lineage of marsupials is traditionally thought to have undergone two major branching events; the first occurring approximately 200 million years ago, giving rise to the theria and monotremata (of which there are only 3 extant species). The therian lineage then branched during the early Cretaceous into the marsupialia and eutheria (placentals) (Tyndale-Biscoe 1973). Marsupials thus demonstrate substantial evolutionary separation from eutherian mammals; marsupials and eutherians are believed to have last shared a common ancestor some 130-150 million years ago (Air *et al* 1971, Archer 1984, Hope RM 1993). This ancient separation of marsupial and eutherian mammals can be exploited to advantage in the study of highly conserved traits (Hope RM 1993). By studying homologous traits of marsupials and eutherians, comparisons can be made of features that have evolved independently of one another for millions of years. Studies in marsupials may, therefore, complement studies done with more conventional laboratory mammals.

It has been generally accepted that at least four anciently diverged marsupial lineages can be identified. The Polyprotodonts (dasyuroids and bandicoots) and Diprotodonts (possums, koalas, wombats, and kangaroos) in Australia, and the Caenolestids and Didelphids in America (Baverstock *et al* 1990). Polyprotodonts and Diprotodonts are estimated to have diverged approximately 40 million years ago (Baverstock *et al* 1990), and although the time of divergence of Australian and American marsupials is

poorly defined, it is likely to have occurred between 60 and 130 million years ago (Hope RM *et al* 1990, Richardson 1988).

Determination of the interrelationships between the major marsupial taxa has proven difficult, and there is still substantial debate relating to both higher order taxonomy and the evolutionary divergence times of marsupials from eutherians and monotremes (Janke *et al* 1997, Kirsch *et al* 1997). Even at the family level, a consensus on classification has not been reached (Cifelli 1993, Hope RM *et al* 1990, Kirsch *et al* 1997, Kirsch & Calaby 1977, Richardson 1988). Table 2.1 shows family classifications based on recent DNA hybridisation studies (Kirsch *et al* 1997). Recent detailed classifications of marsupials to the levels of subfamily and tribe are also available (Kirsch *et al* 1997). Traditional views of evolutionary relationships between monotremes, marsupials and eutherians postulate an early divergence of monotremes. This traditional, or 'Theria hypothesis' approach to marsupial evolution has recently come under question (Janke *et al* 1997), and there is increasing support for the 'Marsupionta hypothesis' which proposes that there is a sister-group relationship between marsupials and monotremes (Janke *et al* 1996). In addition, DNA hybridisation studies by Kirsch *et al* (1997) suggest that no extant marsupial lineage originated before the late Cretaceous and, furthermore, that the American and Australian distinction is misleading. Other groups have also suggested that the classification of marsupials needs re-examination (Janke *et al* 1997, Lee *et al* 1999). Nevertheless, the antiquity of this divergence between the two major mammalian lineages infers that marsupials will exhibit differences when compared to their

eutherian counterparts, and these differences can accordingly be exploited to advantage in genetic and evolutionary investigations.

|    | <b>FAMILY</b>                           |
|----|-----------------------------------------|
| 1  | Peramelidae (bandicoots)                |
| 2  | Thylacomyidae (rabbit eared bandicoots) |
| 3  | Macropodidae (kangaroos and wallabies)  |
| 4  | Burramyidae (pygmy phalangers)          |
| 5  | Phalangeridae (phalangers)              |
| 6  | Pseudocheiridae (ringtail possums)      |
| 7  | Tarsipedidae (honey possums)            |
| 8  | Petauridae (gliding phalangers)         |
| 9  | Acrobatidae (feather-tail gliders)      |
| 10 | Phascolarctidae (koalas)                |
| 11 | Vombatidae (wombats)                    |
| 12 | Microbiotheriidae (monito del monte)    |
| 13 | Notoryctidae (marsupial mole)           |
| 14 | Dasyuridae (dunnarts, native cats)      |
| 15 | Caluromyidae (wooly opossums)           |
| 16 | Didelphidae (opossums)                  |
| 17 | Caenolestidae (shrew opossums)          |

**Table 2.1:** Family classification of extant marsupials (from Kirsch *et al* 1997)

## 2.3 THE REGULATION OF APPETITE AND ENERGY BALANCE IN MARSUPIALS

In comparison to some eutherian species such as rodents and humans, there is relatively little information about the regulation of appetite, energy expenditure or body composition in marsupials. Several studies have examined diet selection, food requirement and feeding activity patterns in marsupials (Goldingay 1990, Nagy *et al* 1988, 1991, Smith 1982), and a few studies have examined alterations in adipose tissue stores (Gordon & Hall 1994, Harris 1987, Morton 1978c, 1980a, Morton *et al* 1983). There are numerous studies which have examined thermoregulation in marsupials, and therefore information relating to metabolic rates, cold acclimation and torpor is readily available (Dawson & Olson 1988, Geiser & Broome 1993, Song *et al* 1997, Wallis 1979, Withers & Hulbert 1988), but the mechanisms by which these processes occur remain unknown.

### 2.3.1 *Appetite regulation in marsupials*

In contrast to the numerous studies examining the physiological mechanisms and neurotransmitters implicated in the regulation of food intake in rodents and humans (see section 1.6), no studies have directly examined the effects of these neurotransmitters on the regulation of food intake in marsupials.

An octapeptide identical to CCK-8 (see section 1.6.3.2) has been identified in the Eastern grey kangaroo (*Macropus giganteus giganteu*), and CCK binding sites are present in the brain of the Brazilian opossum (*Monodelphis domestica*), including the

anterior hypothalamus (Fox *et al* 1990, Johansen & Shulkes 1993, Kuehl-Kovarik *et al* 1993). These studies suggest that CCK developed before marsupials diverged from other mammals and may therefore play a role in the behavioural regulation of food intake in marsupials, as well as in eutherians. However, the effect of CCK on food intake in marsupials has not previously been determined.

Insulin has been purified from the pancreas of the Eastern grey kangaroo and the opossum (*Didelphis albiventris*), showing high homology with human and other mammalian insulins (Treacy *et al* 1989, Yu *et al* 1989). Insulin and glucagon (see section 1.6.3.1) have been detected by immunohistochemical techniques in pancreatic islets of the brush-tailed possum (*Trichosurus vulpecula*) (Reddy *et al* 1986), and glucagon has been purified from the opossum (*Didelphis virginiana*) (Yu *et al* 1989). Opossum glucagon is identical to chicken glucagon, with both differing from the usual mammalian glucagon by a single amino acid substitution (Yu *et al* 1989). It is not known whether pancreatic peptides such as insulin and glucagon affect food intake in marsupials.

Both galanin- and neuropeptide Y-like immunoreactivity (see sections 1.6.4.3 & 1.6.4.7) have been demonstrated in sympathetic vascular neurons of the brush-tailed possum (Morris *et al* 1992), and gastrin like immunoreactivity has been demonstrated in the gastrointestinal tract of a large range of marsupials including the honey possum (*Tarsipes rostratus*), Eastern grey kangaroo, common wombat (*Vombatus ursinus*), and short-nosed bandicoot (*Isodon macrourus*) (Johansen & Shulkes 1993, Takagi *et al* 1990, Yamada *et al* 1989). In addition, serotonin (see section 1.6.3.6),

somatostatin (see section 1.6.3.5), motilin (see section 1.6.4.5) and neurotensin (see section 1.6.3.10) have all been detected by immunohistochemical means in the gastrointestinal tract of several marsupial species (Reddy *et al* 1986, Takagi *et al* 1990, Yamada *et al* 1989), although the effects of these neurotransmitters on food intake have not been determined.

Although the above studies suggest that several neurotransmitters that are likely to be important in appetite regulation of eutheria are also present in marsupials, their effect on food intake in marsupials has not been directly examined. It was hypothesised that as in other mammals, a range of both gastrointestinal and hypothalamic neurotransmitters are likely to be involved in the regulation of food intake in marsupials. The role of several of these neurotransmitters (endogenous opioid peptides, cholecystokinin, bombesin, gastrin releasing peptide, glucagon-like peptide-1, corticotrophin releasing factor and urocortin) on food intake in the marsupial *Sminthopsis crassicaudata* were examined. These studies are presented in chapters 10, 11, and 12.

### **2.3.2      *Thermogenesis in marsupials***

The mechanisms involved in the regulation of thermogenesis and energy expenditure in marsupials are poorly understood. In marsupials, energy metabolism is reported to be 30-35% below that predicted for eutherian mammals; body temperature, heart rate, and nitrogen requirement are also reported to be lower than that of typical eutherian mammals of the same size (Brown & Main 1967, Dawson & Hulbert 1969, 1970, Dawson & Needham 1981, Kinnear & Brown 1967). Thyroid activity, and the

excretion of creatinine (an end product of protein catabolism) have similarly been shown to be lower in marsupials compared to eutheria, and it has been reported that dasyurid marsupials expend less energy for locomotion than eutherians of the same mass (Baudinette *et al* 1976, Bauman & Turner 1966, Fraser & Kinnear 1969). It has recently been shown, that with the exception of certain outlier eutherian orders (Artiodactylata, Lagomorpha, Xenarthra), the basal metabolic rates of marsupials are no less than those of eutherians, when the effects of body size, geographical zone and aridity are considered (Lovegrove 1996). Certain marsupial families may even have higher than average mammalian basal metabolic rates (eg. the Macropodidae). Many marsupials are heterothermic, and large amplitudes of circadian metabolic rhythms have been suggested to account for the perception of low metabolic rates (Lovegrove 1996).

It has also previously been believed that marsupials are less effective at thermoregulation than eutheria, however, it is now known that marsupials can regulate body temperatures as effectively as other mammals (Dawson 1989, McNab 1978, 1980, 1986, Rose *et al* 1990). The mechanisms by which marsupials regulate body temperature are not clearly defined, and the occurrence of brown adipose tissue and non-shivering thermogenesis are controversial (see 2.3.2.1 and 2.3.2.2). Furthermore, the physiological adaptations to cold exposure (apart from the occurrence of torpor) are poorly studied. It was hypothesised that the marsupial *Sminthopsis crassicaudata* would maintain body temperature during cold exposure, facilitated by an increase in food intake and a utilisation of body fat stores. This issue is examined in chapter 13.

Marsupials are born at a rudimentary stage of development; the majority of development occurs in the pouch. At birth, all marsupials are ectothermic; the capacity to generate endogenous heat develops gradually during pouched life, and is associated with changes in insulation and thermal conductance (Gemmell & Cepen 1993, Gemmell & Johnston 1985, Rose 1987). There is little information regarding the mechanisms that control the onset of thermoregulation in marsupials, and, in contrast to most mammals, brown adipose tissue (see sections 1.7.3 and 2.3.2.1) is unlikely to be involved. A recent study performed in this laboratory, however, has indicated that uncoupling protein-2 expression (see section 1.7.3.3) in muscle and perirenal fat increases from birth to 150 days of age in the tammar wallaby (*Macropus eugenii*). This increase in UCP2 is associated with increased oxygen consumption, suggesting a possible mechanism for the onset of thermoregulation in this species (G Wittert & R Baudinette, personal communication).

#### 2.3.2.1 *Brown adipose tissue in marsupials*

There is much controversy as to the presence of brown adipose tissue (BAT) and the occurrence of non-shivering thermogenesis (NST) (see section 1.7.3) in marsupials. Using a combination of electron microscopy and GDP binding, Loudon *et al* (1985) suggested that BAT was present in young Bennett's wallabies (*Macropus rufogriseus*). The majority of studies performed to date, however, suggest that BAT is absent in marsupials (Gemmell & Cepen 1993, Hayward & Lisson 1992, Trayhurn 1993, Ye *et al* 1996). Gemmell and Cepen (1993) failed to find brown fat-like histology in the brush-tailed possum, and Hayward and Lisson (1992) did not find any evidence of BAT in a study of 38 species of marsupial, using microscopic analysis.



Furthermore, attempts to identify UCP1 (the definitive marker of BAT) in marsupials have to date been unsuccessful (Trayhurn 1993).

An interscapular pad of adipose tissue (the primary location of BAT in rodents) is present in the marsupial *Sminthopsis crassicaudata*. It was hypothesised that this tissue contains BAT and that this BAT may be utilised during cold exposure and for arousal from torpor. Chapter 13 provides the first evidence that UCP1 can be detected in the marsupial *S. crassicaudata*, and examines the role of UCP1 in thermoregulation of *S. crassicaudata*.

#### 2.3.2.2 *Non-shivering thermogenesis in marsupials*

In addition to the controversy surrounding the presence of brown fat in marsupials, the role of catecholamines and adrenergic receptors (see section 1.7.3) in the regulation of thermogenesis in marsupials is similarly uncertain. In marsupials, increases in thermogenesis in response to noradrenaline have been reported to occur only in the macropods, for example the potoroo (*Potorous tridactylus*), wallaby (*Macropus rufogriseus*), and bettong (*Bettongia gaimardi*) (Loudon *et al* 1985, Nicol 1978, Nicol *et al* 1997, Ye *et al* 1996). Previous studies in other groups of marsupials, such as the opossum (*Monodelphis domestica*) and the dasyurids (*Antechinus stuartii* and *Dasyuroides byrnei*), have failed to show an increase in thermogenesis in response to noradrenaline (Dawson & Olson 1988, Reynolds & Hulbert 1982, Smith & Dawson 1984). In macropods, the thermogenic response to noradrenaline does not appear to be mediated by  $\beta$ 3-adrenergic receptors (Nicol *et al* 1997);  $\alpha$ 1-adrenergic receptors appear to be more important, at least in the bettong

(Ye *et al* 1996). In recent studies by Nicol *et al* (1997), there was no effect of either noradrenaline or selective  $\beta_3$  adrenergic receptor agonists (ICI D7114 and BRL 35135) on thermogenesis in adult or juvenile brush-tailed possums (*Trichosurus vulpecula*), Brazilian opossums (*Monodelphis domestica*), and adult and juvenile Bennett's wallabies (*Macropus rufogriseus*). It has been suggested that the increase in thermogenesis induced by catecholamines in macropods is mediated via an effect on skeletal muscle (Nicol *et al* 1997).

While it might be expected that non-shivering thermogenesis would be most readily observed in the dasyurids, since they are the smallest of the marsupials (Nicol *et al* 1997), noradrenaline does not increase thermogenesis in *A. stuartii* or *D. byrnei* (Nicol *et al* 1997, Reynolds & Hulbert 1982). A critical review of the manuscript by Reynolds & Hulbert (1982) reveals that although *D. byrnei* clearly showed an increase in thermoregulation during cold acclimation, the effect of noradrenaline on thermoregulation was not directly studied. Recent studies performed in this laboratory have demonstrated an increase in thermogenesis in the dasyurid marsupial *Sminthopsis crassicaudata* in response to noradrenaline (Clements *et al* 1998), providing additional evidence that the sympathetic nervous system plays a role in the regulation of thermogenesis, at least in some marsupials.

#### 2.3.2.3 Diet-induced thermogenesis in marsupials

The occurrence of diet-induced thermogenesis (DIT) (see section 1.7.4) has not been previously reported in marsupials, probably because it has not been evaluated specifically. Recent studies performed in this laboratory have demonstrated that the

marsupial *Sminthopsis crassicaudata* shows an increase in oxygen consumption ( $\text{VO}_2$ ) following food ingestion (Clements *et al* 1998); this is the first direct demonstration of DIT in a marsupial. In addition, it was shown that the magnitude of the increase in  $\text{VO}_2$  was dependent on both the nutritional status of the animal as well as the macronutrient composition of the diet; in non-deprived *S. crassicaudata* the increase in  $\text{VO}_2$  was greater than in 24-hour food deprived animals, and the higher the carbohydrate content of the diet, the greater the thermogenic response (Clements *et al* 1998).

## 2.4 *Sminthopsis crassicaudata* (the fat-tailed dunnart)

*Sminthopsis crassicaudata* is the marsupial model used for the studies presented in this thesis. The University of Adelaide houses a large colony of this marsupial species, and consequently animals were readily available.

### 2.4.1 *Classification, distribution, habitat and morphological variation*

*S. crassicaudata* (the fat-tailed dunnart) is a nocturnal Australian marsupial (body weight 10-20 g), and one of the smallest members of the family Dasyuridae, Fig 2.1. The Dasyuridae are a fairly uniform group taxonomically, with the difference between species being mainly due to size. All members of the Dasyuridae are carnivorous and they occur in a wide range of habitats (Tyndale-Biscoe 1973).

**Figure 2.1:** *Sminthopsis crassicaudata* (the fat-tailed dunnart)  
Photograph courtesy of The Genetics Department, Adelaide University



The distribution of *S. crassicaudata* is ecologically diverse. Animals are found widely, but patchily, distributed across southern and central mainland Australia, extending over south-western Western Australia, South Australia, Victoria, New South Wales and into Southern Queensland (Archer 1981, Godfrey & Crowcroft 1971, Marlow 1965, Morton 1978a). *S. crassicaudata* inhabit mesic, semiarid and arid regions, including grasslands, woodlands, low scrubland and dry stony deserts with clay, sandy or stony substrates. In arid inland habitats, *S. crassicaudata* is known to shelter under stumps and in cracks in the soil. *S. crassicaudata* have been found under rocks in cooler temperate regions, where they construct grass nests and excavate simple tunnels leading to these nests (Morton 1978a, Morton *et al* 1983, Read 1987).

Animals from geographically distant regions differ in many morphological features including foot-pad configuration, body size, pelage colour, ear length, and tail length (Hope RM & Godfrey 1988, Morton & Alexander 1982). Body size remains constant throughout the range of *S. crassicaudata*, but tail and ear length decrease with increasing latitudes. These changes may represent thermoregulatory responses in accordance with Allen's rule; that appendages become smaller in individuals inhabiting colder environments (Morton & Alexander 1982). The impact of ear and tail length on thermoregulation in *S. crassicaudata* has not been studied. Pelage colour is under genetic control and has been shown to lighten with increasing latitudes (Hope RM & Godfrey 1988). Differences in pelage colour have been attributed to camouflage and the avoidance of predation by owls during periods of activity, with only the dorsal pelage colour varying over the species' range, with ventral colour remaining constant. In the northern animals, their sandy colouring allows them to blend in well with their

desert surroundings, whereas southern animals from wetter climates are darker in colour (Hope RM & Godfrey 1988). In nocturnal animals, pelage colour is unlikely to play a role in the regulation of body temperature (Schmidt-Nielsen 1964), although this possibility has not been directly examined in *S. crassicaudata*.

#### 2.4.2 *Diet and food intake*

In the wild, the diet of *S. crassicaudata* consists almost entirely of invertebrates; predominantly spiders, termites and beetles (Morton *et al* 1983). Small reptiles may occasionally contribute to a portion of the diet (Morton *et al* 1983). Because of their insectivorous diet, *S. crassicaudata* relies on an unpredictable food supply that shows seasonal fluctuations in abundance (prey is more abundant during the warmer months). These short-term fluctuations are primarily due to alterations in climatic conditions, and variations in distribution and abundance of prey resulting from changes in vegetation (Morton 1978b).

In contrast to the need for physiological restriction of water usage in many granivorous desert-dwelling rodents, *S. crassicaudata* can subsist without drinking water because of the high water content of their insectivorous diet (Morton 1980b, Nagy *et al* 1988). In *S. crassicaudata*, mean daily water turnovers of 110-190% body water in the wild, and 50-75% body water in the laboratory, have been estimated. These high turnover rates probably reflect the high water content of *S. crassicaudata*'s insectivorous diet, and consequently a lack of selection for water conservation (Morton 1980b). The use of insects as a food source in desert dwelling

small animals may virtually remove the physiological problems of water conservation (Morton 1980b, Nagy *et al* 1988).

Laboratory populations of *S. crassicaudata* have been successfully fed on jellied egg, processed lambs brains, minced beef and vitamin, calcium and iron supplements, tinned pet food, and dry pet food. Mealworm larvae (*Tenebrio molitor*) and crickets can also be used to supplement the diet of captive animals (Bennett *et al* 1982, Ewer 1967, Smith *et al* 1978).

No studies have directly examined the regulation of appetite and food intake in *S. crassicaudata*. In the spring, wild adults have been reported to eat approximately 15 g (fresh mass) of arthropods each day and juveniles around 5 g per day, ie. 80-90% of their own body mass in arthropods each day (Nagy *et al* 1988). Although Ewer (1967) reported an intensive bout of feeding just before dawn, very little other information on food intake and its regulation is available for this marsupial.

The natural alteration of night and day and seasonal changes in day length, have been demonstrated to effect food intake and fat mass in a range of species (Bunning 1967, Morley 1995, Zucker & Stephan 1973). Gender differences in feeding behaviour have also been demonstrated (Nance 1976, Nance *et al* 1976). No previous study has determined feeding patterns in response to either photoperiod or gender in marsupials. In addition, the effect of food deprivation on subsequent food intake has not previously been examined in a marsupial. It was hypothesised that *S. crassicaudata* exhibit alterations in feeding behaviour and fat mass in response to both gender and



photoperiod, and that fasting would induce a subsequent increase in food intake. These studies are described in chapter 4.

The oxidation of metabolic fuels has been demonstrated to affect food intake in mammals (Friedman & Stricker 1976, Friedman *et al* 1986). Both glucoprivation and inhibition of fatty acid oxidation result in increased food intake in rats, mice and a range of other species, however these effects are species specific (DelPrete *et al* 1998, Houpt 1974, Rowland 1978, Smith & Epstein 1969, Stamper & Dark 1997). The effect of metabolic fuels on food intake have not been previously studied in a marsupial species. It was hypothesised that glucoprivation induced by either insulin or 2-deoxy-D-glucose, and inhibition of fatty acid oxidation by mercaptoacetate, would induce an increase in food intake in *S. crassicaudata*. Chapters 8 and 9 respectively examine this issue.

The standard laboratory diet fed to *S. crassicaudata* in these studies consisted of a mixture of commercially available dog and cat food (see section 3.2.1). The standard laboratory diet is substantially lower in calories (1.01 kcal/g) compared to a diet of mealworms (2.99 kcal/g), which more closely approximates the natural insectivorous diet of *S. crassicaudata*. In addition, the standard laboratory diet is lower in fat and protein content than mealworms (see section 3.2.1). Studies in rodents suggest that both the palatability of the diet and subsequent food choice are governed by fat content; higher fat diets tend to be more palatable than lower fat. Higher fat diets also tend to be higher in calories diets (Louis-Sylvestre *et al* 1984). The effects of a high fat, high calorie diet on fat mass, and the regulation of food choice have not

previously been examined in a marsupial. The endogenous opioid peptides (see section 1.6.4.4) have been demonstrated to influence food choice in a number of animal species (Cooper *et al* 1985a,b, Dum *et al* 1983, Levine *et al* 1990), however the effect of opioid peptides on food choice in a marsupial has not previously been determined. It was hypothesised that *S. crassicaudata* would prefer mealworms to their standard laboratory diet, and that peripheral administration of opioid peptide antagonists would influence food choice. These studies are described in chapter 11.

### 2.4.3 Activity

*S. crassicaudata*, like the majority of marsupials, is primarily nocturnally active (Moss & Croft 1988). In the wild, increased activity is strongly associated with light rain (Read 1988), presumably due to increased food availability. Activity is not associated with atmospheric pressure, cloud cover or moonlight, but declines with a decrease in temperature and relative humidity (Read 1988). Male *S. crassicaudata* are more active than females outside breeding periods, with females more active during breeding, presumably because of increased food requirements (Morton 1978b). Laboratory studies have shown that the minimal activity of *S. crassicaudata* during the day coincides with periodic urination and defecation (Crowcroft & Godfrey 1968, Ewer 1967). Peak activity occurs during the first two hours after dark; a series of phases of activity is punctuated by rest periods throughout the night (Ewer 1967). Duration and number of night activity periods shows great variation according to environmental conditions and reproductive condition. Laboratory studies have recently demonstrated that *S. crassicaudata* shows alterations in activity in response to photoperiod; long-day housed males are more active than short-day housed males

(Holloway & Geiser 1996). Differences in the activity of captive females in response to photoperiod have not previously been determined.

#### 2.4.4 *Reproduction*

*S. crassicaudata* is polyoestrous with an oestrous cycle length of approximately 31 days and a gestation period of 13-16 days (Bennett *et al* 1990, Godfrey & Crowcroft 1971). Although more than 10 may be born, a maximum of 10 young can be reared. The young are suckled for about 70 days and, as the mother has an oestrous 1-2 days after suckling ceases, a second litter may be born 80-90 days after the first (Smith *et al* 1978). Females become sexually mature (ie. 1st oestrous) at approximately 91 days, while males mature at approximately 210 days (Bennett *et al* 1990).

Although it has been speculated that breeding in this species may be opportunistic (Ewer 1967, Tyndale-Biscoe 1973), studies by Morton (1978c) have failed to provide evidence of opportunistic breeding, and indicate that *S. crassicaudata* are seasonal breeders. Experiments in wild populations have suggested that the breeding season ranges from June to February, with individual females raising up to two litters per season, some breeding in two seasons (Morton 1978c).

Studies of reproduction in *S. crassicaudata* are more numerous in laboratory animals than in the wild (Bennett *et al* 1982, Godfrey & Crowcroft 1971, Smith & Godfrey 1970, Smith *et al* 1978). In captivity, female *S. crassicaudata* can produce and rear as many as five litters successively and, unlike the situation in the wild, litters can be produced throughout the year via manipulation of lighting regimes (Chesson & Hope

RM 1995). Oestrous cycles cease when animals are housed under short-day photoperiods, and resume under long-day photoperiods (Bennett *et al* 1990). Standard lighting regimes used in captive colonies have been designed to maximise breeding potential and usually consist of 6 months of long-day treatment (16-h light, 8-h dark) followed by 3 weeks of short-day treatment (8-h light, 16-h dark), before being returned to long-day treatment (Bennett *et al* 1990, Smith *et al* 1978). Animals housed continually on long-days will enter anoestrous (Bennett *et al* 1990).

In many mammalian species including rodents, adipose tissue mass fluctuates in response to photoperiod and/or changes in the production of gonadal steroids (Wade & Bartness 1984a,b, Wade *et al* 1985). To date, no studies have directly examined the role of photoperiod or gonadal steroids on fat storage in marsupials. It is hypothesised that the marsupial *S. crassicaudata* will show alterations in both food intake and body fat stores in response to both photoperiod and gonadectomy. These studies are described in chapter 14.

#### 2.4.5 *Caudal fat storage in S. crassicaudata*

*S. crassicaudata*, along with a small number of other marsupial species, are known to store fat in the tail (Morton 1980a, 1978c). Fat-tailed invertebrates other than marsupials can be found in rodentia (Naumov & Lobachev 1975, Walker 1975), primates (Martin 1973, Russell 1975), and the insectivora (Eisenberg & Gould 1970, Walker 1975). Table 2.2 lists some of the small fat-tailed mammals in various animal groups. The localised distribution of fat in large mammals (eg. the fat-tailed sheep), is likely to be advantageous in allowing heat dissipation from the rest of the body

(Schmidt-Nielsen 1964), however, as small marsupials and other small mammals tend to avoid high daytime temperatures, this function is unlikely to be important for them (Morton 1980a), and localisation of caudal fat probably results from other selective pressures. Pond (1978) has shown that in many mammals fat deposition is governed by postural and locomotory factors, and has suggested that fat is stored in the tails of small mammals because in this anatomical location it does not interfere with running or manoeuvrability.

Caudal fat seems to act as a short-term energy reserve in the majority of small vertebrates that exhibit this characteristic. These caudal fat stores are part of a larger fat storage system, and are usually found in small, desert-dwelling insectivorous animals (Morton 1980a). The ability to store large amounts of fat in the tail appears to be of value to animals that live in areas of irregular food supply. For example, lizards such as skinks rely primarily on a diet of invertebrates in the wild, and invertebrate numbers fluctuate widely according to seasonal conditions (Doughty & Shine 1998, Smyth 1974, Taylor 1986). The skinks *Eulamprus tympanum*, *Hemiergis peronii* and *Ctenotus taeniolatus* lack abdominal fat bodies but use the tail as an organ of fat storage (Doughty & Shine 1998, Smyth 1974, Taylor 1986). *H. peronii* stores 40-80% of all extractable fat in the tail (Smyth 1974), and although lipid is distributed throughout the carcass of *C. taeniolatus*, carcass lipid remains unchanged throughout the year, while tail lipid shows distinct seasonal cycles associated with over-wintering and reproduction (Taylor 1986).

| Family                          | Species                                | Common name                           |
|---------------------------------|----------------------------------------|---------------------------------------|
| Dasyuridae (Marsupialia)        | <i>Pseudantechinus macdonnellensis</i> | fat-tailed antechinus                 |
|                                 | <i>Dasykaluta rosamondae</i>           | little red antechinus                 |
|                                 | <i>Planigale gilesi</i>                | paucident planigale                   |
|                                 | <i>Dasycercus cristicaudata</i>        | crest-tailed marsupial mouse, mulgara |
|                                 | <i>Sminthopsis crassicaudata</i>       | fat-tailed dunnart                    |
|                                 | <i>Sminthopsis granulipes</i>          | white-tailed dunnart                  |
|                                 | <i>Sminthopsis hirtipes</i>            | hairy-footed dunnart                  |
|                                 | <i>Sminthopsis macroura</i>            | stripe-faced dunnart                  |
|                                 | <i>Sminthopsis ooldea</i>              | Ooldea dunnart                        |
|                                 | <i>Sminthopsis youngsoni</i>           | lesser hairy-footed dunnart           |
| Didelphidae (Marsupialia)       | <i>Lestodelphys halli</i>              | lestodelpho                           |
| Burramyidae (Marsupialia)       | <i>Cercartetus nanus</i>               | eastern pygmy-possum                  |
|                                 | <i>Cercartetus lepidus</i>             | little pygmy-possum                   |
| Caenolestidae (Marsupialia)     | <i>Rhyncholestes raphanurus</i>        |                                       |
| Microbiotheriidae (Marsupialia) | <i>Dromiciops australis</i>            | monito del monte                      |
| Heteromyidae (Rodentia)         | <i>Microdipodops pallidus</i>          | pale kangaroo mouse                   |
|                                 | <i>Microdipodops megacephalus</i>      | dark kangaroo mouse                   |
| Dipodidae (Rodentia)            | <i>Stylodipus telum</i>                | thick-tailed three-toed jerboa        |
|                                 | <i>Cardiocranius paradoxus</i>         | file-toed pygmy jerboa                |
|                                 | <i>Pygeretmus platyurus</i>            | lesser fat-tailed jerboa              |
|                                 | <i>Salpingotus crassicaudata</i>       | thick-tailed pygmy jerboa             |
|                                 | <i>Salpingotus thomasi</i>             | Thomas' pygmy jerboa                  |
|                                 | <i>Pachyuromys duprasi</i>             | fat-tailed gerbil                     |
|                                 |                                        |                                       |
| Cricetidae (Rodentia)           | <i>Cheirogaleus medius</i>             | fat-tailed dwarf lemur                |
| Lemuridae (Primates)            | <i>Microcebus murinus</i>              | gray mouse lemur                      |
| Tenrecidae (Insectivora)        | <i>Microgale dobsoni</i>               | Dobson's shrew                        |
|                                 | <i>Microgale thomasi</i>               | Thomas' shrew                         |
| Talpidae (Insectivora)          | <i>Condylura cristata</i>              | star-nosed mole                       |

**Table 2.2:** Fat-tailed small mammals (from Morton 1980a)

It appears that caudal fat storage has evolved similarly in several small, desert-dwelling marsupial species, that rely on insects as a food source. These carnivorous marsupials are unable to store food (unlike granivorous rodents), and hence must store energy to survive the unpredictable insect shortages (Morton 1980a). It is important to note that not all desert dwelling insectivorous mammals store fat in their tails, as mechanisms other than fat storage exist that enable these animals to overcome food shortage. For example, *Planigale tenuirostris*, a dasyurid found in the same habitat as *S. crassicaudata*, does not exhibit caudal fat storage (Sauer 1973). In contrast to *S. crassicaudata*, *P. tenuirostris* spends relatively little time foraging for food on the open ground, as its food supply of termites and ants (which do not fluctuate in number or activity to the same extent as non social insects that comprise the majority of *S. crassicaudata*'s diet), is located primarily in a micro-environment partially protected from full climatic factors (Sauer 1973).

*S. crassicaudata* forages for insects at night on the ground surface. Because the habitats of *S. crassicaudata* are open and relatively unprotected from climatic changes, *S. crassicaudata* has to withstand short, unpredictable periods of severe food shortage during cold, wet or windy conditions. *S. crassicaudata* exhibits a number of adaptations in order to overcome short-term variability in food supply; these include high mobility, nest-sharing, repeated breeding within an uncertain optimal period of food supply, and diurnal torpor during winter (Morton 1978b,c,d). Several studies have provided evidence that tail fat is used as an energy source during periods of low food availability; in *S. crassicaudata* deprived of food, tail fat stores decrease as compared to well-fed animals (Godfrey 1968, Ride 1970). In free-living *S.*

*crassicaudata*, there are seasonal changes in the amount of fat stored in the tail which are related to changes in food supply and reproduction (Morton 1978c). During spring and summer (when food is abundant), tail widths decline in both males and females, and during the breeding season, females tend to have thin shrunken tails, suggesting that energy is used for reproduction rather than fat storage. Tail widths increase prior to the onset of the cooler winter conditions, showing a peak just before breeding (Morton 1978c).

Although there are many studies examining the regulation of fat mass in rodents and humans, the regulation of body fat stores in marsupials has not been adequately assessed. The reliability of tail width as an indicator of both tail fat and total fat storage in *S. crassicaudata* has similarly not been studied. It was hypothesised that tail fat stores of *S. crassicaudata* represent total body fat stores, and that lipectomy (fat removal) would induce a subsequent regeneration of fat mass, consistent with the overall regulation of body fat stores (lipostatic hypothesis, see section 1.3). These studies are described in chapter 5.

Leptin, the protein product of the *ob* gene, signals the size of adipose tissue deposits to the hypothalamus (Considine *et al* 1996, and see section 1.5). Leptin decreases food intake, body weight and fat mass in rodents after both central and peripheral administration (Campfield *et al* 1996, 1997). The leptin sequence is highly conserved across species (Ashwell *et al* 1998, Gong *et al* 1996, Zhang *et al* 1994), however there is no molecular information available on leptin in marsupials, and the effect of leptin administration on food intake, body weight and fat mass has not been examined.



It was hypothesised that marsupial leptin would share a similar high homology to the leptin sequence from other species, and that, as in rodents, leptin administration would decrease food intake and fat mass in *S. crassicaudata*. These studies are described in chapters 6 and 7 respectively.

Feeding of a high fat, high calorie diet has been demonstrated to increase fat mass in rodents (Boozer *et al* 1995, Rogers 1985). Increased plasma leptin levels, resulting from increased fat storage, are associated with reduced sensitivity to leptin administration, suggesting partial leptin resistance (Halaas *et al* 1997, Widdowson *et al* 1997). It was hypothesised that a diet of mealworms would induce and increase in both body weight and fat mass in *S. crassicaudata* and that this increased fat mass would induce resistance to the actions of peripherally administered leptin. These studies are described in chapter 7.

#### 2.4.6 *Thermoregulation in S. crassicaudata*

*S. crassicaudata* has a thermoneutral zone from 31.5 to 35.5 °C, which is often higher than the ambient temperatures of its natural environment (MacMillen & Nelson 1969). Early studies of small dasyurid marsupials suggest that they cannot endure temperatures in excess of 40 °C, despite inhabiting areas where such temperatures occur. Exposure of animals to 40 °C results in a steady rise in body temperature to nearly 40 °C, with animals showing signs of distress, such as lying still and breathing fast (Robinson & Morrison 1957). In the wild, *S. crassicaudata* overcomes high temperatures by behavioural means, such as avoiding daylight hours, and also exhibits

a diurnal variation in body temperature, the lowest temperatures being recorded during the middle of the day (Tyndale-Biscoe 1973).

*S. crassicaudata* shows a variety of responses to cold, including huddling, nest sharing, basking, reduced activity and torpor (Wallis 1979). Torpor occurs in the wild after dry and cold nights when food availability is limited and is associated with a progressive decline in metabolic rate (Frey 1991). *S. crassicaudata* does not readily enter torpor in the wild, however, and torpor appears to be reserved for an emergency measure (Morton 1978d, Tyndale-Biscoe 1973). In the laboratory, torpor can be easily induced with fasting and cold exposure (Geiser & Baudinette 1987, Godfrey 1968). As discussed in section 3.2.2, the physiological adaptations to cold exposure in *S. crassicaudata* are examined in chapter 13.

## 2.5 *Overall aims of the study*

Clearly there is little information available on the regulation of body fat stores, food intake and energy balance in marsupials. Furthermore, there are numerous areas of controversy that exist in marsupial physiology, such as the issue of brown fat and its presence/or absence. The aim of these studies was to evaluate the regulation of body fat stores, food intake and energy balance in the marsupial *S. crassicaudata* and to examine some of the mechanisms by which this occurs. The broad hypothesis tested is that there is an integration between the factors regulating food intake, fat storage and energy balance in the marsupial *S. crassicaudata*.

Specific aims of each study are outlined below.

- 1) To establish the feeding behaviour of *S. crassicaudata* in captivity, as well as the effects of gender, photoperiod and food deprivation (Chapter 4).
- 2) To determine in *S. crassicaudata* i) the relationship between tail width, body weight and adipose tissue stores, ii) the effect of tail removal (lipectomy) on energy intake, body weight and remaining adipose tissue stores, and iii) the effect of a high calorie, higher fat diet on body weight and adipose tissue stores (Chapter 5).
- 3) To isolate and sequence cDNA for leptin from adipose tissue of *S. crassicaudata*, to establish the chromosomal localisation of the leptin gene, and to investigate the tissue distribution of leptin mRNA (Chapter 6).
- 4) To determine in *S. crassicaudata* i) the effect of chronic leptin administration on food intake, body weight, fat stores and energy metabolism, and ii) whether leptin administration can prevent a diet-induced increase in adiposity (Chapter 7).
- 5) To determine the effect of insulin-induced hypoglycaemia, and 2-deoxy-D-glucose on food intake in *S. crassicaudata* (Chapter 8).
- 6) To determine the effect of mercaptoacetate induced inhibition of fatty acid oxidation on food intake in *S. crassicaudata* (Chapter 9).

7) To determine the effects of peripheral administration of the peptides corticotrophin releasing factor and urocortin on food intake, thermogenesis and plasma cortisol in the marsupial *S. crassicaudata*, and ii) the receptor/s mediating these effects (Chapter 10).

8) To establish i) whether *S. crassicaudata* prefer the higher calorie and fat diet of mealworms to their standard laboratory diet, and ii) the effect of opioid peptide antagonists selective for  $\mu$ ,  $\delta$ , and  $\kappa$  receptors on food intake and food choice (Chapter 11).

9) To determine the effect of the gastrointestinal peptides cholecystokinin, bombesin, gastrin-releasing peptide and glucagon-like peptide-1 on food intake in *S. crassicaudata* (Chapter 12).

10) To determine in *S. crassicaudata* i) whether brown adipose tissue is present in interscapular fat depots, ii) the response to acute and chronic cold exposure, iii) whether interscapular adipose tissue is used for re-warming from torpor, and iv) the presence of uncoupling protein-2 mRNA expression (Chapter 13).

11) To determine the effects of ovariectomy and photoperiod on adiposity, energy intake and uncoupling protein-2 mRNA expression in *S. crassicaudata* (Chapter 14).

## CHAPTER 3

### GENERAL MATERIALS AND METHODS

All experimental protocols outlined in this thesis were approved by the Animal Ethics Committees of the University of Adelaide (approval # M43/96) and the Royal Adelaide Hospital (approval # 28/97).

#### 3.1 ANIMALS AND HOUSING

##### 3.1.1 *Animals*

*Sminthopsis crassicaudata* were purchased from a breeding colony maintained by the University of Adelaide, Animal Services. Sexually mature adults were used in all experiments so as to avoid the potentially confounding effects of puberty and ageing. Ages ranged from 7-12 months, and from 5-12 months, in males and females respectively. The lifespan of *S. crassicaudata* in captivity averages approximately 1.5 years (personal observations), although there have been reports of animals surviving for over 3 years (Bennett *et al* 1990).

##### 3.1.2 *Housing*

*S. crassicaudata* were housed in well ventilated, windowless rooms that were temperature- and photoperiod-controlled via the use of artificial light. Alternatively, animals were housed in constant temperature cabinets (Quantec Precision

Engineering, SA, Australia). Ambient temperature could be varied according to requirements, however animals were usually housed at  $24 \pm 1^{\circ}\text{C}$  unless otherwise specified. In all experiments that required individual identification and measurements, animals were housed individually in cages of 10.5 x 12.5 x 26 cm, containing sawdust, and a cardboard cylinder for cover. After completion of these experiments, animals were returned to group living conditions. Groups of 3-5 animals could be housed in polypropylene cages of 44 x 28 x 16 cm containing sawdust and a plastic nesting box (20 x 10 x 5 cm) loosely filled with shredded paper. For experiments that did not require individual measurements, animals were housed in groups. These animals were distinguished via the use of ear marking. All cages were cleaned weekly.

### 3.1.3 *Variation in day length*

Two regimes of day length were used: long-days (LD; 16 hours light, 8 hours dark) and short-days (SD; 8 hours light, 16 hours dark) representing summer and winter day lengths respectively. These day lengths were chosen on the basis of lighting cycles utilised in the breeding of *S. crassicaudata* (Bennett *et al* 1990). In some of the experiments performed early in my research, SD housed animals had a 9 hours light, 15 hours dark regime, because of the breeding requirements of the colony in which they were initially housed.

## 3.2 DIETS

### 3.2.1 *Standard laboratory diet*

Animals were fed *ad libitum* on the standard laboratory diet, unless otherwise stated. This diet consisted of a mixture of water, *Woofs* dry dog food (Ridley Agri-Products, SA, Australia) and *Whiskas* cat food (Uncle Ben's, Vic, Australia), as described by Bennett *et al* (1982), in the ratio of 100: 40: 45 respectively. The standard laboratory diet had an energy content of 1.01 kcal/g (4.2 KJ/g), and it contained approximately 20% fat, 25% protein, 55% carbohydrate by dry weight (manufacturer's information), with approximately 70% water. Caloric content of the diet was confirmed by bomb calorimetry.

### 3.2.2 *Mealworms*

Live mealworms (*Tenebrio molitor* larvae) were obtained from S.A. Mealworm Breeders (Wingfield, SA, Australia) and provided *ad libitum* as stated in the experimental protocols. The energy content of mealworm larvae was 2.99 kcal/g (12.5 KJ/g), and they contained approximately 30% fat, 60% protein, 10% carbohydrate by dry weight (The Mealworm Company, Sheffield, UK), with approximately 55% water. Caloric content of mealworms was confirmed using bomb calorimetry.

### 3.2.3 *Water*

Water was provided *ad libitum* via sipper bottles at all times.

### 3.3 MEASUREMENTS

#### 3.3.1 *Tail width*

Tail width (mm) was measured at the widest point to the nearest 0.1mm, by drawing the tail through circular holes of known diameter until an exact fit was obtained.

#### 3.3.2 *Energy intake (food intake)*

Intake of standard laboratory diet was determined by placing a plastic bowl containing a weighed amount of the diet into each cage. The bowl was re-weighed at each time point. To control for weight loss through evaporation, laboratory diet was also placed in empty cages and weighed at each time point. When calculating the energy intake for an animal at each time point, an adjustment was made for weight loss through evaporation.

Mealworms were presented in a small jar and intake of mealworms was quantified by weighing the jar at each time point.

Food intake was expressed as kilocalories eaten, or kilocalories eaten per gram of body weight.

#### 3.3.3 *Body temperature*

Body temperature (°C) was measured by inserting a thermocouple probe (MC-87, dual channel thermometer, TPS, Qld, Australia) 5 mm into the rectum.



### 3.3.4 *Metabolic parameters*

Individual *S. crassicaudata* were placed in a clear perspex cylindrical chamber (22 cm long x 8 cm in diameter), which was located in a large, photoperiod-controlled, constant temperature cabinet (Quantech, SA, Australia). The air outflow from the chamber was connected to an infra-red gas (O<sub>2</sub> and CO<sub>2</sub>) analyser (Thoroughbuild Electronics, SA, Australia). The expired gas was initially passed through a column containing drierite crystals, (CaSO<sub>4</sub>), to remove water vapour. This dry air was then pumped through the gas analyser at a known rate by a pump connected to a flow meter (calibrated to approximately 500 ml/min). Fresh air entered the chamber from small inlet ports situated at the opposite end to the outlet port. 'Baseline' O<sub>2</sub> consumption and CO<sub>2</sub> production were calculated within 30 minutes of the introduction of the animal into the chamber, and minimum consumption was calculated using the Haldane equation modified for an open flow system (Frappell & Daniels 1991).

Briefly, as:

$$VO_2 = VIO_2 - VEO_2 \text{ in ml/min (at STPD)} \quad (i)$$

$$\text{and} \quad VI = \frac{VE \times [1 - (FECO_2 + FEO_2)]}{1 - (FICO_2 + FIO_2)} \quad (ii)$$

substituting back and replacing VI, then

$$\frac{VO_2 = VE \times [1 - (FECO_2 + FEO_2)] \times FIO_2 - VE FEO_2}{1 - (FICO_2 + FIO_2)} \quad (iii)$$

(where I = Inspired, E = Expired and F = Fraction of total gas, VE = the flow rate through the analysers).

VCO<sub>2</sub> was determined by:

$$\text{VCO}_2 (\text{ml/min}) = (\text{VECO}_2 - \text{VICO}_2) \times \text{VE}$$

VICO<sub>2</sub> was taken to be zero. Both VO<sub>2</sub> and VCO<sub>2</sub> were expressed as ml/h/g body mass after first converting the measured values to standard temperature and pressure dry (STPD) by multiplying by:

$$\frac{P_1 \times T_s}{P_s \times T_1}$$

$$P_s \times T_1$$

where P<sub>1</sub> = atmospheric pressure, P<sub>s</sub> = standard pressure (760 mmHg)

T<sub>1</sub> = experimental temperature, T<sub>s</sub> = standard temperature (0 °C)

The Respiratory Quotient (RQ) was calculated as:

$$\frac{\text{VCO}_2}{\text{VO}_2}$$

### 3.4 BLOOD SAMPLING

Blood was sampled by puncturing the orbital sinus using a 29- gauge needle and withdrawing blood into a 1 ml syringe. Up to approximately 300 µl of blood was collected on each occasion, depending on requirements.

#### 3.4.1 *Blood glucose concentration*

Quantitative measurement of blood glucose concentration (mmol/l) was performed using a commercial glucometer (Medisense Waltham, MA, USA), immediately following sampling. The glucometer allows rapid measurement of blood glucose, using an electrochemical detection technique (Cass *et al* 1984). Within-run precision was 5.6% at 2.22 mmol/l, 3.7% at 5.5 mmol/l and 3.4% at 8.17 mmol/l.

#### 3.4.2 *Plasma free fatty acid concentration*

Following sampling, blood was spun at 4 °C for 15 minutes and the plasma stored at -20 °C. The quantitative determination of free (non-esterified) fatty acids in plasma (mEq/l) was performed using a WAKO NEFA-C test kit (Novachem, Vic, Australia). The assay uses an original *in vitro* enzymatic colorimetric method that is loosely based on other extraction methods (Duncombe 1964, Itaya & Ui 1965). The assay was carried out according to the manufacturer's instructions and the optical density of the samples at 550 nm determined on an Abbott Biochromatic Analyser 100 (Abbott Laboratories, CA, USA). The interassay coefficient of variation was 2.7% at 0.33 mEq/l, 1.1% at 0.62 mEq/l and 1.1% at 0.99 mEq/l.

#### 3.4.3 *Plasma cortisol concentration*

Following sampling, blood was spun at 4 °C for 15 minutes and the plasma immediately assayed for cortisol. Plasma cortisol was measured using a fluorescent polarisation immuno-assay (Abbott Laboratories, IL, USA). The interassay coefficient of variation was 8.7% at 108 nmol/l, 4.0% at 449 nmol/l and 4.7% at 837 nmol/l. Assays were performed using a TDx analyser (Abbott Laboratories, IL, USA).

### 3.5 DRUG PREPARATION AND ADMINISTRATION

#### 3.5.1 *Leptin*

*Purified r-met human leptin*, 5 mg/ml (Amgen, CD, USA), was stored at -70 °C and diluted in sterile phosphate-buffered saline, pH 7.2 (PBS), immediately prior to use. Leptin and PBS vehicle were administered intraperitoneally (IP).

#### 3.5.2 *2-deoxy-D-glucose and insulin*

*2-deoxy-D-glucose* (2-DG) (Sigma Chemical Company, NSW, Australia), a metabolic blocker of glucose utilisation, and regular *insulin* (Novo Nordisk Pharmaceuticals, NSW, Australia), were stored at 4 °C and diluted to the appropriate concentration in 0.9% sodium chloride immediately prior to use. 2-DG and insulin were administered intraperitoneally (IP).

#### 3.5.3 *Mercaptoacetate*

*Mercaptoacetate* (MA; thioglycolic acid, sodium salt, Sigma Chemical Company, NSW, Australia), an inhibitor of fatty acid oxidation, was stored in powdered form at -20 °C until use. MA was diluted to the appropriate concentration in 0.9% sodium chloride immediately prior to use. MA, or 0.9% saline alone, were administered intraperitoneally (IP).

#### 3.5.4 *Opioid peptide antagonists*

The selective  $\mu$  opioid antagonist *Naloxone* (naloxone hydrochloride, Sigma Chemical Company, NSW, Australia), the selective  $\delta$  antagonist *Naltrindole* (naltrindole

hydrochloride, Sigma Chemical Company, NSW, Australia), and the selective  $\kappa$  antagonist *nor-binaltorphimine dihydrochloride* (nor-BNI, Research Biochemicals International, MA, USA) were stored in powdered form at -20 °C, and diluted to the appropriate concentration in 0.9% sodium chloride immediately prior to use. Naloxone and Naltrindole were administered subcutaneously (s.c.) immediately before re-feeding; nor-BNI was administered intraperitoneally (IP), 120 minutes before re-feeding. The receptor specificity of these antagonists is discussed in chapter 11.

### 3.5.5 *Gastrointestinal peptides*

The gastrointestinal peptides *cholecystokinin octapeptide* (CCK; fragment 26-33 amide, non-sulphated, Sigma Chemical Company, NSW, Australia), *bombesin* (BBS; Peninsula Laboratories, California, USA), *gastrin releasing peptide* (porcine) (GRP; Silenus Laboratories, Vic, Australia), and *glucagon-like peptide-1* (GLP-1; human, synthetic, preproglucagon 72-108, Sigma Chemical Company, NSW, Australia) were stored in powdered form at -20 °C, and diluted to the appropriate concentration in 0.9% sodium chloride immediately prior to use. All drugs were administered intraperitoneally (IP).

### 3.5.6 *Urocortin, corticotrophin releasing factor and antalarmin*

*Urocortin* (rat) (UCN; Peninsula Laboratories, Merseyside, UK) and *corticotrophin-releasing factor* (ovine) (CRF; American Peptide Company, CA, USA) were stored in powdered form at -80 °C and diluted to the appropriate concentration in 0.9% saline immediately prior to administration. Both UCN and CRF were administered intraperitoneally (IP).

The CRF 1 receptor antagonist *antalarmin* (ANT; N-Butyl-N-ethyl-[2,5,6,-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]amine) was synthesised by Dr. Kenner C. Rice (Medicinal Chemistry, NIH, Bethesda, USA) (Webster *et al* 1996), and was a gift from Dr. George P. Chrousos (Developmental Endocrinology, NIH, Bethesda, USA). ANT was initially prepared as a 100 mg/ml stock solution (Bornstein *et al* 1998, Webster *et al* 1996). Five hundred microliters of absolute ethanol was added to 100 mg of ANT and heated for 4 minutes at 65 °C with occasional vortexing. Five hundred microliters of cremaphor EL (BASF Australia, Vic, Australia) was then added and the solution was heated for 4 minutes at 65 °C until the compound went easily into solution with vortexing. Four milliliters of hot sterile distilled water (dH<sub>2</sub>O) was then added and the solution vortexed thoroughly. A 'control' stock solution was prepared as above, without adding any ANT. Stock solutions were stored at 4 °C. On the day of the experiment, the ANT stock was warmed on a heating block at 65 °C, vortexed thoroughly, and diluted to the required concentration in hot dH<sub>2</sub>O. The injected 'control' was prepared from the 'control' stock solution using identical volumes to those required for the ANT dilution.

### 3.6 SURGERY

Surgery was performed in a warm, well ventilated room to prevent rapid loss of body heat by anaesthetised animals. Heating pads were placed below the animals both during surgery and during recovery from anaesthetic. Following surgery, all animals were observed closely.

### **3.6.1**            *Tail removal*

Sham surgery (anaesthesia only) or tail removal was performed under light inhalation anaesthesia with ethrane (approximately 1.5%) (Enflurane Inhalation Anaesthetic, Abbott Australasia, Kurnell, Australia), carried by a 1:1 mixture of oxygen and nitrous oxide at 1L/min. Tails were removed at the base using a sharp sterile blade, and the wound was immediately cauterised. Animals were weighed before and immediately after surgery. Removed tails were weighed and analysed for fat content using the triglyceride assay described in section 3.7.1 (Salmon & Flatt, 1985).

### **3.6.2**            *Ovariectomy*

Ovariectomy (or sham ovariectomy) was performed under light inhalation anaesthesia with Fluothane (approximately 1.5%) (Fluothane Inhalation Anaesthetic, ICI Australia Operations, Vic, Australia), carried by a 1:1 mixture of oxygen and nitrous oxide at 1L/min. Surgery was performed via a ventral incision. The ovarian arteries were ligated, the ovaries excised and the wound closed with sterile sutures and treated with Betadine antiseptic solution (Faulding Pharmaceuticals, SA, Australia). All procedures were identical for the sham ovariectomised animals, except that the ovarian arteries were not ligated and the ovaries remained intact.

### 3.7 CHARACTERISATION OF ADIPOSE TISSUE

#### 3.7.1 *Analysis of fat content using a triglyceride assay*

Measurement of total triglyceride was performed using an established method (Salmon & Flatt 1985). Tail and body triglyceride contents were determined separately. Briefly, tails and bodies were digested at 60 °C for 2 days in freshly made alcoholic KOH (1 part ethanol to 1 part 30% KOH). Forty milliliters of KOH was used to digest the bodies and 12 ml for the tails. Following digestion, the volume was increased to 100 ml for bodies and 25 ml for tails, using 50% ethanol. After mixing, 0.5 ml was removed to a microfuge tube to which 540 µl of 1M MgCl<sub>2</sub> was then added. Samples were vortexed, placed on ice for 10 minutes and then spun at 13 000 rpm for 15 minutes at 4 °C. The quantitative determination of total triglyceride in the resulting supernatant was performed using the Unimate 5 triglyceride test kit (Roche Products, NSW, Australia). This assay uses an *in vitro* enzymatic colorimetric method (Kohlmeier 1986) and was carried out according to the manufacturer's instructions. Optical density of the samples at 500 nm was determined using a Cobas Fara centrifugal analyser (Roche Diagnostics, Zurich, Switzerland). A molecular weight of 885 was assumed per triglyceride molecule (Salmon & Flatt 1985).

#### 3.7.2 *Electron microscopy*

Interscapular and tail fat were quickly immersed in chilled (4 °C) fixative (pH 7.2) containing 3% glutaraldehyde, 4% paraformaldehyde, 4% sucrose and 4% polyvinylpyrrolidone in 6 mmol/L phosphate buffered saline, and cut into 1 mm cubes. Tissues remained in the same fixative for a further 18 hours and were then post-fixed



in 1% osmium tetroxide, dehydrated through a series of graded ethanols and embedded in Spurr's resin (polymerised at 70 °C for 72 hours). Ultra-thin sections (silver-gold colour) were cut from the resin-embedded blocks on a Reichert Om U2 ultra-microtome using a diamond knife. After examining 1 micron thick sections and selecting the appropriate areas, the ultra-thin sections were mounted on copper (200 mesh) grids, stained with 2% alcoholic uranyl acetate and lead citrate respectively for 20 minutes, and then examined in a Jeol x 100 electron microscope at 80 kV (Kumaratilake *et al* 1991).

### 3.7.3 *Biochemical analysis of interscapular adipose tissue*

Interscapular adipose tissue was weighed, and homogenised in a 250 mM sucrose buffer containing 200 mM EDTA and 1 mM hepes (pH 7.2). An aliquot was taken for determination of total tissue protein (Lowry *et al* 1951). A further aliquot was delipidated and assayed for DNA content, using the fluorescent dye Hoechst 33258 (Brunk *et al* 1979). The remaining tissue homogenate was used to prepare mitochondria (Trayhurn *et al* 1987).

#### 3.7.3.1 *GDP binding*

Mitochondrial protein (100 µg) was incubated in 200 nM <sup>3</sup>H labelled guanosine diphosphate (GDP), 0.1 mCi/ml of <sup>14</sup>C labelled sucrose and 2µM cold GDP, in a buffer containing 100 mM sucrose, 20 mM HEPES, 1 mM EDTA, 10 mM choline chloride, 4 µM rotenone and 0.8% fatty acid free bovine serum albumin. After shaking at room temperature for 7 minutes, the tubes were centrifuged for 3 minutes at 13 000 rpm, and the supernatant removed. The pellet was solubilised in 100 µl of tissue solubliser

(NCS-II, Amersham) for 60 minutes and a 50 µl aliquot transferred to 5 ml of scintillation fluid, neutralised and counted for radioactivity (Trayhurn *et al* 1987). The assay was performed in triplicate with a single tube containing 200 µM GDP used to calculate non-specific binding.  $^{14}\text{C}$  sucrose was used to correct for entrapment of supernatant (Trayhurn *et al* 1987).

#### 3.7.3.2 *Immunological identification of uncoupling protein-1 (UCP1)*

Mitochondria were solubilised in 1% SDS at 100 °C for 5 minutes, and 10 µg of protein per lane applied to a 20% homogenous mini-gel (Phast System, Pharmacia Biotech, Hong Kong). After electrophoresis, the proteins were transferred to nitrocellulose at 70 °C for 30 minutes. UCP1 was detected by probing the blot with rabbit anti-ground squirrel UCP1 serum (Milner & Trayhurn 1990). A horse-radish peroxidase-conjugated sheep-anti rabbit IgG antiserum (Silenus, Vic, Australia) was used to detect antibody-antigen complexes, which were visualised by enhanced chemiluminescence (Amersham, NSW, Australia).

### 3.8 LABORATORY TECHNIQUES

#### 3.8.1 *RNA extractions and agarose gel electrophoresis*

*S. crassicaudata* were killed by rapid decapitation. The tissues were quickly removed, frozen in liquid nitrogen, and then stored at -80 °C. Total RNA was extracted from tissue by a modification of the 'cesium chloride method' described by Chirgwin (1979). Briefly, tissue was homogenised in guanidium thiocyanate (GTC) and the

resulting homogenate was then layered onto a cushion of 5.7M cesium chloride (CsCl) and centrifuged at 65 000 rpm for 18 hours at 20 °C. Pellets were resuspended in DEPC water and ethanol precipitated at -80 °C overnight (Sambrook *et al* 1989). The RNA concentration of the extracts was determined from the absorbance at 260nm.

15-25 µg/lane of total RNA was applied to a 1.4% denaturing agarose gel, and the components separated according to size by gel electrophoresis. Ethidium bromide staining was used to confirm equal RNA loading for all blots. RNA was then transferred overnight to Hybond-N<sup>+</sup> nylon transfer membrane (Amersham, NSW, Australia) by capillary blotting and fixed to the membrane by cross-linking with UV light.

### 3.8.2 *Probes*

#### 3.8.2.1 *Uncoupling protein-1 (UCP1)*

The sequence for the 27mer UCP1 oligonucleotide was obtained from previously published work (Trayhurn *et al* 1994), and is complementary to the conserved region of the rat and cattle UCP1 genes. The oligonucleotide was synthesised commercially (Bresatec, SA, Australia) with a single digoxigenin (DIG) ligand (Boehringer Mannheim, Vic, Australia) at the 5' end. The sequence of the oligonucleotide used is indicated in brackets (6GACTTTGGCGGTGTCCAGCGGGAAGGT-3'). Northern blotting was carried out as stated in section 3.8.4.

### 3.8.2.2 *Uncoupling protein-2 (UCP2)*

Human UCP2 cDNA, containing pBluescript SK (-) plasmids, were a gift from Dr. O. Boss (Division of Endocrinology, Harvard Medical School, MA, USA). The UCP2 cDNA probe (GenBank Accession #U82819) (Boss *et al* 1997a) was obtained by digesting the plasmid with XbaI and XhoI to produce a 1105 bp cDNA insert. The probe was labelled with [ $\alpha$ - $^{32}$ P] dCTP (Bresatec, SA, Australia) to a specific radioactivity of approximately  $1 \times 10^9$  dpm/ $\mu$ g DNA using a Gigaprime DNA labelling kit (Bresatec, SA, Australia). Northern blotting was carried out as stated in section 3.8.3.

### 3.8.2.3 *Leptin cDNA probe*

A 1.8kb *S. crassicaudata* leptin cDNA fragment from clone pSMFL2, as described in chapter 6, was used to detect leptin mRNA on Northern blots. The probe was labelled with [ $\alpha$ - $^{32}$ P] dCTP (Bresatec, SA, Australia) to a specific radioactivity of approximately  $1 \times 10^9$  dpm/ $\mu$ g DNA using a Gigaprime DNA labelling kit (Bresatec, SA, Australia). Northern blotting was carried out as stated in section 3.8.3.

### 3.8.3 *Northern blotting using $^{32}$ P labelled DNA probes*

Pre-hybridisation was performed for 30 minutes at 68 °C in QuickHyb hybridisation solution (Stratagene, Integrated Sciences, NSW, Australia) according to the protocol supplied by the manufacturers. Briefly, RNA blots were hybridised for 4 hours at 65 °C in QuickHyb containing the probe at  $1.25 \times 10^6$  counts/ml. Post-hybridisation washes were performed at 50 °C and consisted of two 5 minute washes in a solution of 2 X SSC/0.1% sodium dodecyl sulfate (SDS), and one 15 minute wash in 0.1 X

SSC/0.1% SDS. Blots were then exposed to Kodak X-Omat AR film (Integrated Sciences, NSW, Australia) for 2-14 days at -80 °C.

#### **3.8.4 Northern blotting using DIG labelled oligonucleotides**

Pre-hybridisation was performed at 42 °C for 2-4 hours in a modified 'church' buffer (Church & Gilbert 1984) containing 7% SDS, 50% formamide, 5 X SSC, 2% blocking reagent (Boehringer Mannheim, Vic, Australia), 0.1% *N*-lauroylsarcosine, and 50 mM sodium phosphate (pH 7.0). Hybridisation was at 42 °C overnight in the pre-hybridisation solution together with the UCP1 DIG labelled oligonucleotide (25-50 ng/ml). Post-hybridisation washes were carried out as follows: (i) 2 X SSC/0.1% SDS for 15 minutes at room temperature (RT) (2 times), and (ii) 0.1 X SSC/0.1% SDS for 20 minutes at 48 °C (2 times). Following post-hybridisation washes, membranes were rinsed at RT for 3 minutes in 'washing buffer' (0.1M maleic acid, 0.15M NaCl, 0.3% Tween 20, pH 7.5) and then processed at RT, as described in the Boehringer Mannheim pack insert. Briefly, membranes were blocked by incubation with 1% blocking reagent (Boehringer Mannheim, Vic, Australia), 0.1M maleic acid, and 0.15M NaCl pH 7.5 for 60 minutes, then incubated in the blocking buffer for 30 minutes with a polyclonal antibody against DIG (Fab fragment; diluted 1:10 000), conjugated to alkaline phosphatase (Boehringer Mannheim, Vic, Australia). The membrane was washed twice for 20 minutes in washing buffer, then transferred to 'detection buffer' 0.1 M Tris/HCL, 0.1 M NaCl, 50 mM MgCl<sub>2</sub> (pH 9.5) for 2 minutes. Finally, membranes were immersed in detection buffer containing the chemiluminescent substrate CDP-Star™ 1:100 (Boehringer Mannheim, Vic, Australia) for 5 minutes. Following incubation with the chemiluminescent substrate, membranes

were exposed to Hyperfilm ECL (Amersham, NSW, Australia) for between 15 minutes and 24 hours at RT.

### **3.8.5      *Quantitation of gene expression following Northern blotting***

The intensity of the bands was determined using Sigma Scan Image (Jandel Scientific, Australia), and corrected for loading by measurement of 18S ribosomal RNA band intensity.

## **3.9            STATISTICAL ANALYSIS**

Data are expressed as raw values, or mean  $\pm$  SEM where appropriate. Statistical analyses were performed by 1- and 2-way analysis of variance (ANOVA) with repeated measures. Unless stated otherwise, multiple comparison procedures were performed at individual time points using the post-hoc Student-Newmann-Keuls test or paired/un-paired Student's t-tests. Pearson's correlation was used to determine the relationship between any two variables. Any animals that died during experiments were not included in statistical analyses. Statistical analyses were carried out using the Sigma Stat software package (Jandel Scientific, Australia). A P value of  $< 0.05$  was considered significant in all analyses.

## CHAPTER 4

**FEEDING PATTERNS IN *S. CRASSICAUDATA*: EFFECTS OF GENDER, PHOTOPERIOD AND FASTING****4.1 SUMMARY**

A number of studies have investigated the regulation of food intake in different mammalian species, but little is known about the regulation of feeding in marsupials. In this study the effects of gender, photoperiod, and fasting on food intake, body weight and tail width (an indicator of fat stores) in the marsupial *S. crassicaudata* were characterised. Studies were carried out in both males and females, maintained under long-day (LD; 16-h light, 8-h dark) and short-day (SD; 9-h light, 15-h dark) light regimes. Circadian food intake was determined by measuring total food intake during both the light and dark cycle. In a separate experiment food intake was measured at 0.5, 1, 2, and 3 hours after lights off. Groups of animals were exposed to 24 and 36 hour periods of food deprivation, and subsequent food intake determined at 0.5, 1, 2, 4, 6, and 24 hours. The effect of a 24-hour fast on blood glucose and plasma free fatty acid levels was determined in male animals housed under a LD light regime.

In both LD and SD males and females, approximately 82% of food intake occurred during the dark phase ( $P < 0.0001$ ), and there was no gender or photoperiod effect. Females maintained under SD's consumed approximately 25% less over a 24-hour

period ( $P < 0.005$ ) than either females under LD's and males under both SD's or LD's, primarily due to a reduction in food intake during the light phase. However, this reduction in food intake was not associated with a decrease in either body weight or tail width. LD females, LD males, SD females, and SD males ate approximately 38%, 20%, 36%, and 42% respectively of their total 24-hour food consumption in the first 3 hours of darkness. Following both 24- and 36-hour fasts, total food intake in the subsequent 24 hours increased ( $P < 0.001$ ) by approximately 2 fold in all groups, with no gender or photoperiod effect. In both 24- and 36-hour deprived groups however, SD females ate 25% and 34% less respectively ( $P < 0.05$ ) than LD females in the first 6 hours after re-feeding. Following 24 or 36 hours of fasting, mean body weight decreased by about 13% and 18% respectively ( $P < 0.01$ ). Tail width decreased an average of 5.5% ( $P < 0.05$ ) in all groups of animals after the 36-hour fast, but after the 24-hour fast tail width was only observed to decrease in LD animals ( $P < 0.05$ ). In LD housed male *S. crassicaudata*, fasting for 24 hours induced a 12% decrease ( $P < 0.001$ ) and a 120% increase ( $P < 0.02$ ) in blood glucose and plasma free fatty acids respectively. These observations establish that in *S. crassicaudata* both photoperiod and gender influence food intake and fat mass, and that fasting is associated with metabolic alterations that may effect subsequent food intake.

## 4.2 INTRODUCTION

As a pre-requisite to subsequent studies which have examined regulation of food intake in the marsupial *Sminthopsis crassicaudata*, the feeding behaviour of this



marsupial under laboratory conditions was determined. To evaluate the effects of gender and photoperiod, studies were performed in animals of both sexes, housed under either long- or short-day photoperiods.

The natural alteration of night and day influences many physiological and behavioural functions in animals (Bunning 1967). For example, rats are nocturnally active mammals that consume approximately 80% of their food in the dark (Zucker 1971), while many species of bird are active primarily during the day (Grimm 1957). Like many rodents, and a range of other mammals including bats, *S. crassicaudata* is a nocturnal mammal; studies in wild and laboratory populations have demonstrated that the majority of activity (and therefore presumably food intake) occurs during the dark (Crowcroft & Godfrey 1968, Ewer 1967, Morton 1978b, Moss & Croft 1988). Despite several studies on activity patterns in the wild and under laboratory conditions, there is very little direct information on feeding patterns and food intake of *S. crassicaudata*.

As well as daily variations in feeding resulting from light/dark cycles, animals are also subjected to seasonal changes in day length; it has been demonstrated in numerous species that substantial variations in food intake occur in the wild in response to these environmental alterations (Morley 1995, Zucker & Stephan 1973). For example, the Siberian hamster (*Phodopus sungorus*) decreases food intake when exposed to short-day lengths in a laboratory setting (Bartness *et al* 1986). Similarly, in the meadow vole (*Microtus pennsylvanicus*) there is a decrease in food consumption on short-day photoperiods (Dark *et al* 1983). In contrast, food intake in golden hamsters

(*Mesocricetus auratus*) does not vary in response to photoperiod (Wade & Bartness 1984b). The effect of photoperiod on food intake and body weight in *S. crassicaudata* is discussed further in chapter 14.

Gender differences in feeding behaviour have been demonstrated in a range of mammalian species. For example, although male rats eat more than females, long term adjustments in feeding behaviour usually occur more rapidly in females (Nance 1976, Nance *et al* 1976, Van Hest *et al* 1988). Gender differences in food intake result, at least in part, from differences in gonadal hormone secretion and this issue is also examined in chapter 14. No studies have evaluated whether there are gender differences in food consumption in *S. crassicaudata*, either in the wild, or under laboratory conditions. It has, however, been demonstrated that outside breeding periods male *S. crassicaudata* are more active than females (Morton 1978c), and it is accordingly likely that food intake would be greater in males during this time, as a result of increased energy requirements.

By quantifying the feeding behaviour of *S. crassicaudata* in the laboratory, the effects of various challenges, similar to those that may occur in the wild, can be determined. For example, the ability of *S. crassicaudata* to adapt to food deprivation is of interest as the availability of food may be limited in wild populations, affecting 'normal' feeding patterns. Because there is marked climatic variation in their natural habitat, it is likely that *S. crassicaudata* has to withstand short periods of severe food shortage on an irregular basis (Morton 1978c). The effect of food deprivation on subsequent food intake varies among different species. In rats, food intake increases substantially

following fasting (Armstrong *et al* 1980), whereas in the Siberian hamster food deprivation for periods as long as 48 hours fails to induce an increase in food intake (Bartness *et al* 1995). The golden hamster shows little or no post-fast food compensation and this has been attributed to hoarding of food, or perhaps hibernation during periods of food scarcity (Silverman & Zucker 1976).

The aim of these studies was to establish the feeding behaviour of *S. crassicaudata* in captivity, as well as the effects of food deprivation, gender and photoperiod.

## 4.3 MATERIALS AND METHODS

### 4.3.1 *Experimental animals and diets*

Adult male and female *S. crassicaudata* housed under either a long-day (LD; 16-h light, 8-h dark) or short-day (SD; 9-h light, 15-h dark) photoperiod, lights off 14:30h, were used as specified in the experimental protocols below. All animals were fed standard laboratory diet *ad libitum*, except where stated.

### 4.3.2 *Experimental protocols*

Study 1: *To establish the circadian feeding rhythm of S. crassicaudata in response to both gender and photoperiod*

LD and SD housed *S. crassicaudata* of both sexes ( $n = 7$  per group) were presented with a weighed amount of laboratory diet at lights on. The amount of food was then weighed at lights off, and again at lights on. This study was repeated over a 4 day

period. Mean data from the 4 day experimental period were combined for statistical analyses.

Study 2: *To determine food consumption immediately after the onset of the dark phase in *S. crassicaudata*, in response to both gender and photoperiod*

LD and SD housed *S. crassicaudata* of both sexes ( $n = 6$  per group) were presented with a weighed amount of laboratory diet at lights off. Food intake was subsequently measured at 0.5, 1, 2, 3, and 24 hours. The experiment was repeated one week later and mean data combined for statistical analyses.

Study 3: *The effect of 24- and 36-hour food deprivation on food intake, body weight, and tail width*

LD and SD housed *S. crassicaudata* of both sexes ( $n = 6$  per group) were deprived of food for 24 or 36 hours. Food intake was measured at 0.5, 1, 2, 4, 6 and 24 hours after re-feeding. The timing of the experiments was such that in each group re-feeding occurred at the commencement of the dark cycle. The experiment was repeated one week later and mean results combined for statistical analyses.

Study 4: *The effect of 24-hour food deprivation on blood glucose and plasma free fatty acids*

LD housed male *S. crassicaudata* ( $n = 7$ ) were deprived of food, commencing at the onset of the dark phase. Twenty four hours later, blood was sampled from the orbital sinus of each animal (see section 3.4). Blood glucose levels were immediately determined (see section 3.4.1), and plasma was retained for free fatty acid

measurements (see section 3.4.2). Animals were then fed *ad libitum* with standard laboratory diet for 24 hours; immediately after this, blood was sampled for the same measurements.

#### 4.3.3 *Statistical Analyses*

Analyses were carried out as stated in section 3.9.

### 4.4 RESULTS

#### 4.4.1 Study 1: Circadian food intake

Approximately 82% of total energy intake occurred during the dark phase in both LD ( $F_{2,40} = 30.3$ ,  $P < 0.0001$ ) and SD ( $F_{2,41} = 26.1$ ,  $P < 0.0001$ ) males and females, Fig 4.1. There was a significant effect of photoperiod on energy intake in females ( $F_{1,41} = 9.5$ ,  $P < 0.005$ ), but not males. Total energy intake over 24 hours was  $0.67 \pm 0.04$  and  $0.50 \pm 0.05$  kcal/g in the LD and SD females respectively. In males, energy intake over 24 hours was  $0.67 \pm 0.03$  and  $0.67 \pm 0.04$  kcal/g in LD and SD housed groups respectively, Fig 4.1.

#### 4.4.2 Study 2: Food intake immediately after the onset of the dark phase

LD females ate more ( $F_{3,47} = 6.91$ ,  $P = 0.001$ ), whereas SD females ate less ( $F_{3,47} = 4.88$ ,  $P = 0.007$ ) than LD males and SD males respectively, in the first 3 hours of darkness. Total energy intake over 3 hours was  $0.17 \pm 0.02$  and  $0.14 \pm 0.02$  kcal/g in the LD and SD females respectively. In males, energy intake over 3 hours was  $0.10 \pm$

0.03 and  $0.18 \pm 0.03$  kcal/g in LD and SD housed groups respectively, Fig 4.2. LD females, LD males, SD females, and SD males ate approximately 38%, 20%, 36%, and 42% of their total 24 hour food consumption in the first 3 hours of darkness.

#### 4.4.3 Study 3: Effect of food deprivation

After both 24- and 36-hour food deprivation, body weight decreased in all animals ( $P < 0.01$ ), Table 4.1. The magnitude of the decrease was greater in 36- as opposed to 24-hour food deprived animals ( $P < 0.05$ ) in all groups apart from the LD housed males, where the magnitude of the decrease was similar in both 24- and 36-hour fasted animals. Following the 24-hour fast, in all groups body weight returned to pre-fast values within 24 hours after re-feeding. However, following the 36-hour fast, body weight failed to return to baseline in either the SD housed males ( $P < 0.02$ ) or females ( $P < 0.05$ ) within 24 hours of re-feeding, Table 4.1.

After the 24 hour fast, tail width decreased in both LD males ( $P < 0.05$ ) and females ( $P < 0.05$ ), but did not change significantly in SD animals. However, when the period of fasting was increased to 36 hours, there was a reduction in tail width in all animals ( $P < 0.05$ ), Table 4.1. Following the 24-hour fast, tail width returned to baseline within 24 hours of feeding in all groups except the LD females ( $P = 0.04$ ). Following the 36-hour fast, tail width failed to return to baseline within 24 hours of feeding in any of the four groups; LD ( $P = 0.02$ ) and SD ( $P = 0.002$ ) housed males, LD ( $P = 0.02$ ) and SD ( $P = 0.04$ ) housed females.

During re-feeding, 24-hour food intake (kcal/g body weight) increased by approximately two fold in all groups of animals, when compared to non fasted controls, ( $P < 0.001$ ); there was no significant effect of either gender or photoperiod on total food intake, Fig 4.3. During the first 6 hours of feeding, all animals ate progressively rather than at one time point, and SD females ate less than LD females during this time ( $F_{1,71} = 5.7$ ,  $P < 0.05$ ), Fig 4.3A. Similar results were evident in both 24- and 36-hour fasted animals, Fig 4.3A,B.

#### 4.4.4 Study 4: Effect of a 24-hour fast on blood glucose and plasma free fatty acids

24-hour food deprivation induced a decrease in blood glucose from  $4.61 \pm 0.41$  to  $4.04 \pm 0.38$  mmol/l ( $P = 0.0009$ ), and an increase in plasma FFA from  $0.25 \pm 0.04$  to  $0.55 \pm 0.08$  mEq/l ( $P = 0.015$ ), Fig 4.4.

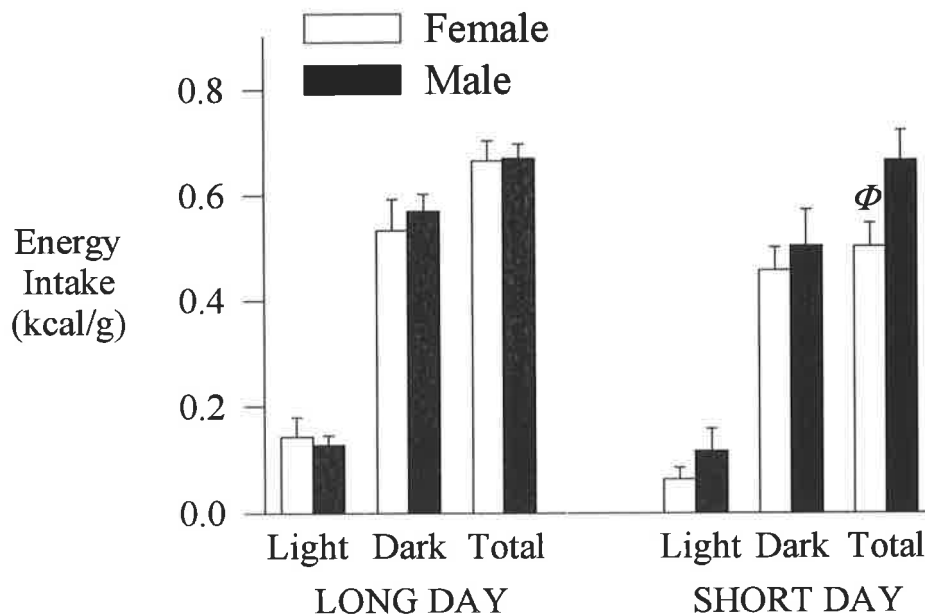


**Figure 4.1:** Energy intake (kcal/g body weight) over 24 hours (showing light-dark feeding) in male and female *S. crassicaudata* with *ad libitum* food supply, under LD and SD conditions (means  $\pm$  SE of 7 animals per group). In all groups the majority of food was consumed during dark hours ( $P < 0.0001$ ). SD females ate less ( $\phi P < 0.01$ ) than any of the other groups over 24 hours.



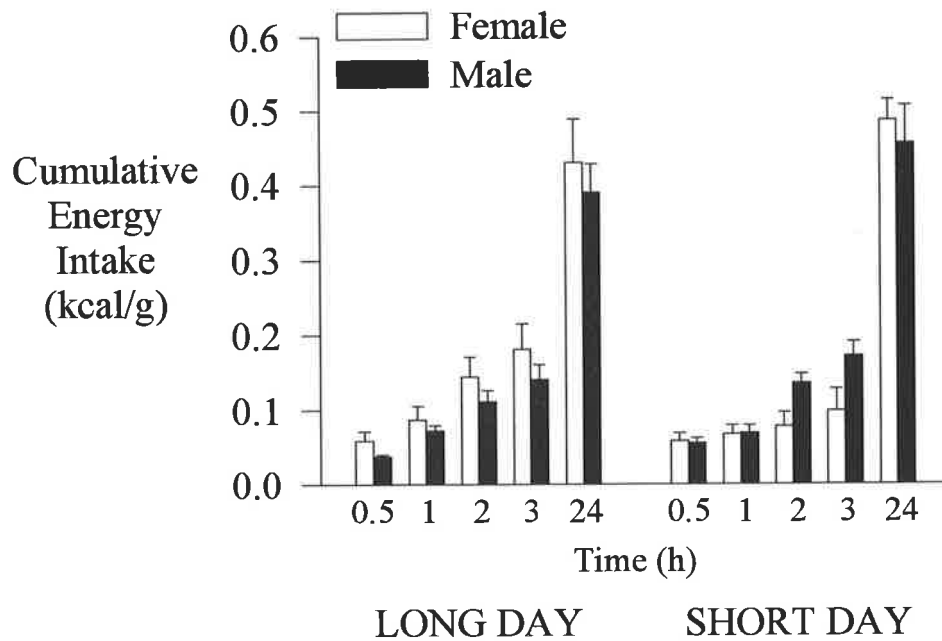


**Figure 4.2:** Energy intake (kcal/g body weight) commencing at the onset of the dark phase in male and female *S. crassicaudata* with *ad libitum* food supply, under LD and SD conditions (means  $\pm$  SE of 6 animals per group). LD females, LD males, SD females, and SD males ate approximately 38%, 20%, 36%, and 42% of their total 24-h food intake respectively, in the first 3 hours of darkness.

|                   | Body Weight (g)  |                 |                    | Tail Width (mm)  |                 |                    |
|-------------------|------------------|-----------------|--------------------|------------------|-----------------|--------------------|
|                   | Before 24-h fast | After 24-h fast | 24-h after feeding | Before 24-h fast | After 24-h fast | 24-h after feeding |
| <b>LD Males</b>   | 14.25 ± 0.47     | 12.37 ± 0.34 †  | 14.40 ± 0.67       | 4.38 ± 0.11      | 4.04 ± 0.10 *   | 4.23 ± 0.12        |
| <b>LD Females</b> | 15.80 ± 0.55     | 13.63 ± 0.47 †  | 15.40 ± 0.59       | 4.13 ± 0.18      | 3.75 ± 0.18 *   | 3.93 ± 0.24 *      |
| <b>SD Males</b>   | 16.49 ± 0.42     | 14.82 ± 0.40 †  | 16.60 ± 0.64       | 4.67 ± 0.09      | 4.50 ± 0.11     | 4.62 ± 0.13        |
| <b>SD Females</b> | 15.51 ± 0.56     | 14.12 ± 0.30 †  | 16.10 ± 0.79       | 4.42 ± 0.20      | 4.21 ± 0.19     | 4.18 ± 0.20        |
|                   | Before 36-h fast | After 36 h fast | 24-h after feeding | Before 36-h fast | After 36-h fast | 24-h after feeding |
|                   | Before 36-h fast | After 36 h fast | 24-h after feeding | Before 36-h fast | After 36-h fast | 24-h after feeding |
| <b>LD Males</b>   | 11.31 ± 0.46     | 9.60 ± 0.10 †   | 11.18 ± 0.57       | 3.62 ± 0.09      | 3.38 ± 0.10 *   | 3.44 ± 0.13 *      |
| <b>LD Females</b> | 16.46 ± 0.53     | 13.25 ± 0.33 ‡  | 16.16 ± 0.80       | 4.17 ± 0.11      | 3.88 ± 0.11 *   | 3.97 ± 0.18 *      |
| <b>SD Males</b>   | 18.17 ± 0.59     | 14.83 ± 0.42 ‡  | 17.48 ± 0.84 *     | 4.66 ± 0.11      | 4.46 ± 0.12 *   | 4.20 ± 0.18 *      |
| <b>SD Females</b> | 17.32 ± 0.56     | 14.12 ± 0.30 ‡  | 16.71 ± 0.45 *     | 4.43 ± 0.38      | 4.19 ± 0.13 *   | 4.40 ± 0.19 *      |

**Table 4.1:** Effects of 24- and 36-h food deprivation on body weight and tail width in LD and SD housed male and female *S. crassicaudata*. Values are means ± SE of 6 animals per group. LD, long day; SD, short day. \*  $P \leq 0.05$ , †  $P < 0.01$ , ‡  $P < 0.001$ , compared to pre-fast values.

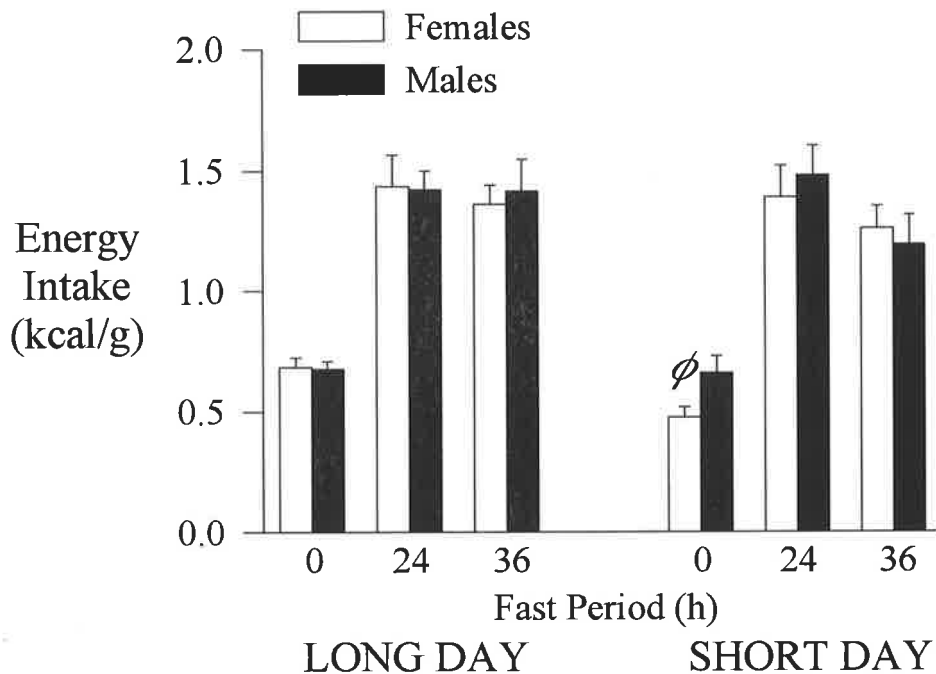


**Figure 4.3:** Total 24-h energy intake (kcal/g body weight) in male and female *S. crassicaudata* housed under both LD and SD photoperiods; with *ad libitum* food supply (0), and after 24-h (24) and 36-h (36) food deprivation (means  $\pm$  SE of 6-7 animals per group). Food intake increased in all groups following both 24- and 36-h food deprivation ( $P < 0.0001$ ). Under conditions of *ad libitum* food supply, SD females ate less than SD males, LD males and LD females ( $\phi P < 0.01$ ).

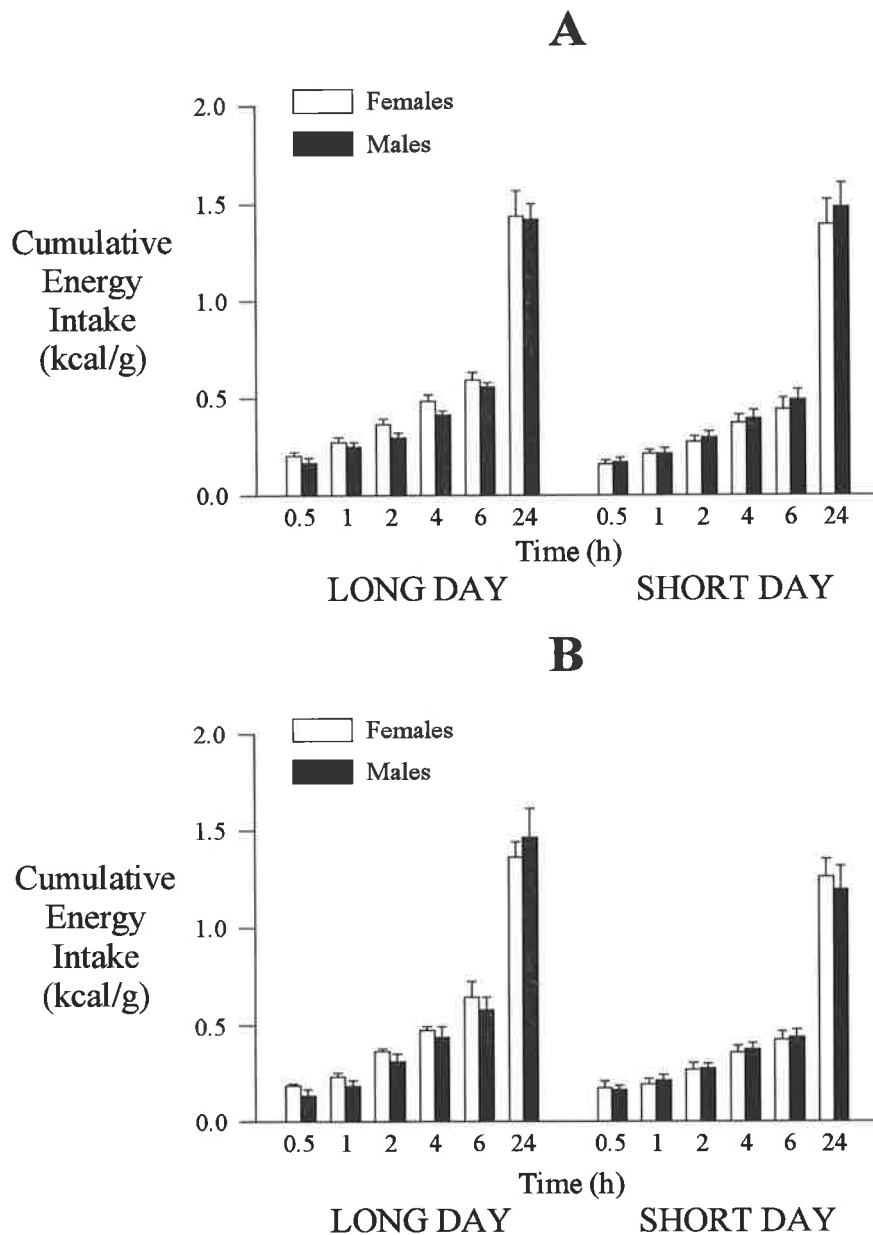


**Figure 4.4:** Cumulative energy intake (kcal/g body weight) over 24-h in male and female *S. crassicaudata* housed under LD and SD photoperiods, and deprived of food for (A) 24 hours, and (B) 36 hours (means  $\pm$  SE of 5-6 animals per group). Photoperiod had no effect on food intake in males, however, SD females consumed less than LD females during the first 6 hours ( $P < 0.05$ ).

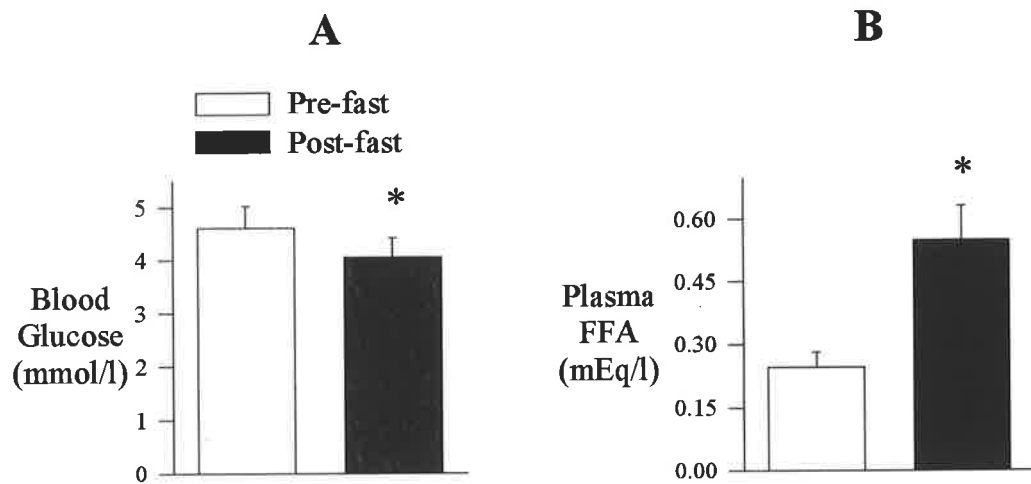


**Figure 4.5:** The effect of a 24-hour fast on (a) blood glucose (mmol/l), and (b) plasma FFA (mEq/l), in male *S. crassicaudata* housed under a LD photoperiod (means  $\pm$  SE of 6-7 animals per group). Fasting decreased blood glucose ( $P = 0.0009$ ) and increased plasma FFA ( $P = 0.015$ ). \*  $P < 0.02$ .

## 4.5 DISCUSSION

These studies demonstrate that *S. crassicaudata* is predominantly a nocturnal feeder; approximately 82% of total food is ingested during the dark period and 35% of total night time intake is eaten during the first 3 hours after lights off. It has been established previously that *S. crassicaudata* are active throughout the night with short rest periods, and that an intensive bout of feeding occurs just before lights on (Ewer 1967). Similar activity patterns have been observed in the carnivorous daysurid marsupial *Phascogale tapoatafa* (Cuttle 1982).

These data also show that there is a photoperiod-dependent effect on food intake in female, but not male *S. crassicaudata* in captivity. Food intake was similar in LD and SD males, but females on SD photoperiods ate less than LD females and less than both LD and SD males over a 24-hour period. This observation is of interest since, theoretically, those animals under short-days have a longer period of darkness in which to eat. The reduced food intake of SD females, however, was primarily due to a reduction in food intake during the light phase. In the wild, *S. crassicaudata* feeds mainly on insects (Morton 1978c) and hence food abundance is reduced greatly during colder months (Stratham 1982). Short days are associated with winter conditions when food is scarce. This, however, does not provide an explanation for these findings, because under conditions of captivity, food is abundant. In laboratory populations, under natural day-lengths, the majority of matings occur between early August and March (Smith *et al* 1978), when (in the southern hemisphere) days are beginning to get longer. One possible explanation is that the increased food intake in

LD females represents a response to an increased energy requirement during the breeding season, when days are longer.

Despite the reduced food intake in SD females there were no significant differences in either body weight or tail width between SD females and LD females following a 5 week period on either day length, suggesting energy conservation occurs as a result of increased metabolic efficiency and/or a decrease in physical activity. Subsequent to the completion of this study, other researchers have demonstrated that, in captivity, male *S. crassicaudata* housed under SD photoperiods show a decrease in the level of physical activity, without changes in basal metabolic rate (Holloway & Geiser 1996). Preliminary studies in our laboratory have been unable to replicate these findings. LD housed male *S. crassicaudata* placed under SD for 3 weeks did not show any alterations in the level of physical activity (H. Turnbull, personal communication). The measurement of activity levels in female *S. crassicaudata* in response to photoperiod remains to be determined, and the effect of photoperiod on activity in male *S. crassicaudata* housed in this laboratory remains to be verified.

The above findings contrast with those of Bartness *et al* (1986, 1995) who showed in Siberian hamsters that while food intake was less under short when compared to long photoperiods, this decrease was associated with weight loss and was a prerequisite for torpor. *S. crassicaudata* does not readily undergo torpor unless subjected to extreme environmental conditions, such as a marked limitation in food availability (Frey 1991, Morton 1980a). Furthermore, in contrast to the Siberian hamster, in which food intake is lower in both males and females under short-days, a reduction in food intake

only occurred in female *S. crassicaudata*. This finding suggests that the effect of photoperiod on feeding behaviour is likely to be linked to seasonal variation in reproduction and alterations in gonadal steroid levels; this issue is further explored in chapter 14 .

As discussed in chapter 5, a unique feature of *S. crassicaudata* is that the tail is a major site for fat storage. Morton (1978c) reported that, in the wild, males have greater stores of fat in their tails than females at all times of the year, and that in both sexes tail width decreased throughout the breeding season, increased in autumn, and subsequently decreased throughout winter and the subsequent breeding season. These observations suggest that energy is used for reproduction rather than fat storage during warmer months. By contrast, the current study did not show differences in tail widths between animals housed on different photoperiods. This is presumably because of the relative abundance of food available to the animals in captivity as compared to variable food availability in the wild.

Following both 24- and 36-hour fasts, food intake was increased by about two fold when compared to non-fasted animals. These observations demonstrate that unlike the Siberian or golden hamster, *S. crassicaudata* exhibit food compensation after fasting. A paucity of food is a common factor associated with diverse climatic conditions, such as those found in south-central Australia and other areas inhabited by this species. A reduction in food availability is likely to be associated with increases in both the amount of time spent searching for food and subsequent food intake. Interestingly, in the Chinese hamster (*Cricetulus griseus*), an animal which like *S.*



*crassicaudata* lives predominantly in arid conditions, feeding does not increase following fasting, suggesting that their feeding drive is constant and not affected by time of day or degree of hunger (Billington *et al* 1984). This is clearly not the case with *S. crassicaudata*.

Following a 24-hour fast, tail width decreased in LD but not in SD females, whereas 36-hour fasting was associated with a reduction in tail width in all groups. These findings indicate that tail fat stores are utilised as an energy source under conditions of food deprivation. Observations that in SD females longer periods of food deprivation were required to decrease tail width, and that SD females ate less than LD females in the first 6 hours of re-feeding after both 24- and 36-hour deprivation, suggest that even under conditions of limited food availability, there is a relative conservation of energy in SD females. The mechanisms by which this energy conservation is accomplished are not known. The potential role of gonadal steroids is investigated in chapter 14.

It has been suggested that levels of circulating metabolites play an important role in feeding (Langhans & Scharrer 1992). As in the majority of mammals studied to date (DiBattista 1983, Scharrer & Langhans 1986, Schneider *et al* 1988, Strubbe *et al* 1988), this study has shown that in *S. crassicaudata* blood glucose levels fall and there is a rise in plasma free fatty acids following a fast. It is possible that, as in some other mammals, alterations in glucose and free fatty acid levels may be important in the regulation of food intake in *S. crassicaudata*. The role of glucoprivation and fatty

acid oxidation on food intake in *S. crassicaudata* is examined further in chapters 8 and 9 respectively.

## CHAPTER 5

**CHARACTERISATION OF TAIL FAT IN *S. CRASSICAUDATA*:  
EFFECT OF LIPECTOMY AND DIET****5.1 SUMMARY**

Although there are many studies examining the regulation of fat mass in rodents and humans, the regulation of body fat stores in marsupials has not been adequately assessed. This study has demonstrated that tail fat stores account for ~ 25% of total body fat in the marsupial *S. crassicaudata*. Tail width was correlated strongly with both body weight ( $r = 0.70$ ,  $P < 0.0001$ ) and tail triglyceride content ( $r = 0.78$ ,  $P < 0.0001$ ), suggesting that tail width can be used as a measure of overall adiposity. Removal of the tail (~ 25% lipectomy) induced an initial decrease in body weight of *S. crassicaudata* ( $P < 0.0001$ ), followed by a restoration of body weight which was not associated with increased food intake. Lipectomised animals regained fat mass subcutaneously, and at completion of the study total fat mass (as measured by triglyceride content) was no different between lipectomised and intact animals. After a period of 24-hour food deprivation, body weight decreased to a similar extent in both lipectomised and intact animals. ( $P < 0.05$ ), and following re-feeding, body weight returned to pre-fast values within 24 hours in both groups.

When fed a higher calorie and fat diet of mealworms in contrast to standard laboratory diet, *S. crassicaudata* gained weight ( $P < 0.0001$ ) and tail width increased ( $P < 0.0001$ ), suggesting an increase in fat mass. After 21 days on the mealworm diet, body weight returned to baseline, corresponding with a decrease in energy intake per gram of body weight. Tail width however was maintained at higher levels, indicating some overall conservation of fat mass.

These studies show that tail width is a reliable measure of overall fat mass in *S. crassicaudata*. The lipectomy study demonstrates that fat stores are defended below a minimum point. In contrast, the upper limit of body fat mass appears to be more flexible.

## 5.2 INTRODUCTION

Caudal (tail) fat storage occurs in a range of animals including some rodents, primates and marsupials (see table 2.2). *S. crassicaudata*, is one of a number of small marsupials that store fat in the tail (see section 2.4.5). In *S. crassicaudata*, this caudal fat store has been proposed to act as a short-term energy reserve, due to the unpredictable nature of the availability of arthropods which make up their diet in the wild (Morton 1980a, Morton *et al* 1983). It is not known, however, how these fat stores are regulated. Previous studies carried out on wild populations of *S. crassicaudata* have demonstrated seasonal changes in tail fat stores; fat stores increased during warmer months when food was abundant, and decreased during the

breeding season as energy was utilised for reproduction (Morton 1978c). In these studies, tail width was used as the indicator of tail fat storage, although the reliability of this measure has not been assessed. There is data relating to the regulation of fat stores in rodents and humans, but it is not known how fat stores are regulated in marsupials.

The lipostatic hypothesis proposed by Kennedy (1953) suggests that the amount of fat in the body is signalled to the brain either by hormonal or neural signals. As a result, alterations in food intake and energy expenditure occur in order to restore energy balance (see section 1.3). Evidence for the lipostatic hypothesis is partly based on studies of the effects of surgical removal of adipose tissue (lipectomy) on body fat stores (see section 1.3.1). However, the dramatic and stressful nature of the surgical procedures required to achieve lipectomy have been criticised and are undoubtedly responsible for many of the conflicting results observed (Weigle 1994). In *S. crassicaudata*, tail removal, a relatively non-invasive procedure, could potentially be used to assess the effects of lipectomy.

The results of lipectomy studies in rodents suggest that fat mass is clearly defended around a minimum 'set-point'. This concept is supported by studies in rodents which show that fat mass lost during food deprivation is regained when food is again available (Bjorntorp & Yang 1982, Yang *et al* 1990). In contrast, the maximum body fat mass is less rigorously defended; both high fat diets, and over-feeding promote obesity in rodents, and body fat mass is subsequently maintained at a higher level (Boozer *et al* 1995, Rogers 1985, Rolls *et al* 1980). Although still controversial, some

studies in rodents and humans demonstrate that dietary fat increases adiposity independent of total caloric intake (Boozer *et al* 1995, Danforth Jr 1985, Dreon *et al* 1988, Eck *et al* 1992, Romieu *et al* 1988). Weight gain resulting from consumption of a high-fat diet may be the result of the high caloric density and/or lower satiating properties of fat compared to other macronutrients (Golay & Bobbioni 1997, Westerterp *et al* 1996).

Little is known about the effect of diet composition on weight gain in marsupials. *S. crassicaudata* clearly prefer to eat mealworms (which more closely approximate their natural insectivorous diet) as compared to their standard laboratory diet (see chapter 11). Mealworms have approximately three times the caloric content of laboratory diet and are higher in fat (30% as opposed to 20% fat by dry weight), (see section 3.2).

The aims of this study were to determine in *S. crassicaudata*: i) the relationship between tail width, body weight and adipose tissue stores, ii) the effect of tail removal (lipectomy) on energy intake, body weight and remaining adipose tissue stores, and iii) the effect of a high calorie, higher fat diet on body weight and adipose tissue stores.

## 5.3 MATERIALS AND METHODS

### 5.3.1 *Experimental animals and diets*

In study 1 (see 5.3.2), equal numbers of adult male and female *S. crassicaudata* were used for the initial determination of tail and body triglyceride content. All subsequent studies used males. Animals were housed under a long-day light regime (LD; 16-h light, 8-h dark), and fed *ad libitum* with standard laboratory diet unless stated. Mealworms were provided where stated in the experimental protocols. Food intake is expressed as kcal/g body weight or total kcal.

### 5.3.2 *Experimental protocols*

#### Study 1: Characterisation of tail fat in *S. crassicaudata*

In order to determine the fat content of the tail in *S. crassicaudata*, 6 adult males and 6 adult females were killed by rapid decapitation. The tail was removed at the base using a sharp blade. A triglyceride assay, as described in section 3.7.1 (Salmon & Flatt 1985), was used to determine the triglyceride content of the tail in relation to that of the body for each animal.

Groups of male *S. crassicaudata* housed under LD ( $n = 27-72$ ) were subsequently used to obtain measurements of tail width (mm) (see section 3.3.1), and total body weight (g), in relation to blood glucose (mmol/l) (see section 3.4.1), and plasma free fatty acid concentration (mEq/l) (see section 3.4.2). In addition, groups of animals were killed by rapid decapitation, their tails removed at the base, and the weight of the removed tail determined ( $n = 43$ ). Tail triglyceride content ( $n = 42$ ), body triglyceride

content minus the tail ( $n = 24$ ), and total triglyceride content ( $n = 24$ ) were measured as above. All visible sub-cutaneous adipose tissue was removed from the carcass of 31 animals and its wet-weight (g) determined.

Study 2: Effect on lipectomy on body weight, energy intake and fat stores in *S. crassicaudata*

Twelve adult male *S. crassicaudata* housed under LD, were separated into 2 groups so that in each group the previous 24-hour energy intake was matched (kcal eaten/g body weight). In one group ( $n = 6$ ), tails were removed and subsequently analysed for triglyceride content (see section 3.7.1). The other group ( $n = 6$ ) underwent anesthesia only. Surgery and sham surgery were performed as outlined in section 3.6.1. Subsequent daily food intake was recorded every day for 44 days. On *day 33*, all food was removed from cages and animals were fasted for 24 hours. Subsequent 24-hour energy intake was recorded after re-feeding. On *day 45*, all animals were killed by rapid decapitation. Bodies and tails were analysed separately for fat content using a triglyceride assay as above.

Study 3: The effect of a preferred (high calorie, higher fat) diet on body weight, energy intake, body temperature and fat stores in *S. crassicaudata*

Adult male *S. crassicaudata* housed under LD were divided into two groups of similar initial body weight ( $n = 5-7$  per group). One group was fed standard laboratory diet only, and the other mealworms, for 42 days. Body weight and food intake were measured daily, and tail width was measured every second day.



### 5.3.3 *Statistical analyses*

Statistical analyses were performed as outlined in section 3.9. After tail removal (lipectomy), changes in body weight were analysed by multivariate ANOVA following matrix transformation of the data.

## 5.4 RESULTS

### 5.4.1 *Characterisation of tail fat*

Tail triglyceride stores accounted for  $24.2 \pm 2.6\%$  and  $25.2 \pm 2.5\%$  of total body triglyceride in female and male animals respectively ( $n = 6$  per group).

Table 5.1 shows mean values obtained for each of the measured parameters. Body weight (g), tail weight (mg), tail triglyceride content (mg) and body fat content (g), showed significant relationships with tail width (mm), Fig 5.1. In addition, significant relationships were found between tail width, body weight, and a range of other parameters outlined in table 5.2. There was no relationship between tail width and blood glucose or free fatty acid concentration. In addition, there was no relationship between body weight and blood glucose concentration, table 5.2.

### 5.4.2 *Effect on lipectomy on body weight, energy intake and tail fat stores*

#### 5.4.2.1 *Body weight*

Body weights of the two groups of animals were similar prior to tail removal ( $14.8 \pm 0.6$  and  $13.5 \pm 0.8$  g in the lipectomised and intact groups respectively). Following tail

removal or sham surgery body weight fell in both groups of animals ( $F = 57.41$ ,  $P < 0.0001$ ), however the fall in body weight was greater in the animals without tails compared to the intact animals ( $F = 21.50$ ,  $P < 0.001$ ). Body weight recovered in both groups of animals ( $P < 0.05$ ), and there was no difference in the rate of recovery or final body weights, Fig 5.2A. Following 24-hour food deprivation on *day 33*, body weight decreased significantly ( $14.8 \pm 0.5$  g to  $12.8 \pm 0.3$  g,  $P < 0.005$  and  $13.7 \pm 0.5$  g to  $12.3 \pm 0.4$  g,  $P < 0.05$ ) in the lipectomised and intact animals respectively. The magnitude of the decrease in body weight was not significantly different between the two groups of animals. Following re-feeding, body weights in both groups of animals returned to pre-fast values within 24 hours, Fig 5.2A

#### 5.4.2.2 *Energy intake*

There was no overall difference in total cumulative energy intake between the lipectomised ( $25.4 \pm 1.0$  kcal/g) and sham ( $28.1 \pm 2.22$  kcal/g) animals; energy intake was similar in both groups at all times, Fig 5.2B. Following 24-hour food deprivation on *day 33*, energy intake over the next 24 hours increased by 76 % ( $0.81 \pm 0.2$  to  $1.1 \pm 0.7$  kcal/g,  $P < 0.05$ ) in the lipectomised animals and by 72% ( $0.5 \pm 0.9$  to  $0.9 \pm 0.1$  kcal/g) in the intact animals, with no significant difference between them, Fig 5.2B.

#### 5.4.2.3 *Fat stores*

On *day 45*, total body triglyceride content (excluding tail fat) was greater in the lipectomised animals ( $517.34 \pm 47.28$  mg) compared to the intact animals ( $363.40 \pm 25.85$  mg) ( $P < 0.02$ ), Fig 5.3. Triglyceride content of the tails of the lipectomised animals, at the time of tail removal, was  $100.5 \pm 12.8$  mg and in the tails of the intact

animals, at the end of the study, triglyceride content was  $102.12 \pm 12.04$  mg. Total triglyceride content of the intact animals was  $465.52 \pm 31.37$  mg, which was not significantly different from the lipectomised animals.

### 5.4.3 *Effect of a high calorie diet on energy intake, body weight, and tail fat stores*

#### 5.4.3.1 *Energy intake*

Cumulative energy intake (kcal) did not differ significantly between the mealworm fed group when compared to the laboratory diet fed group. On *day 42*, cumulative food intake was  $697 \pm 30.6$  kcal (approximately  $209 \pm 9.2$  kcal as fat) and  $735 \pm 82.7$  kcal (approximately  $147 \pm 16.5$  kcal as fat) in the mealworm and laboratory diet fed groups respectively, Fig 5.4A.

When energy intake was expressed as kcal/g body weight, a significant difference in cumulative energy intake between the mealworm fed and laboratory diet fed groups was found (diet x day  $F_{42,429} = 4.56$ ,  $P < 0.0001$ ). This difference was the result of a decreased energy intake per gram of body weight in the mealworm as compared to the laboratory diet group over the last 21 days of the experiment, Fig 5.4B. Cumulative energy intake on *day 42* was  $38.9 \pm 1.8$  kcal/g and  $47.2 \pm 4.6$  kcal/g in the mealworm and laboratory diet fed groups respectively. Cumulative energy intake did not differ between the two groups over the first 21 days of the experiment and was  $22.7 \pm 0.89$  kcal/g and  $23.9 \pm 2.03$  kcal/g in the mealworm fed and laboratory diet groups respectively, Fig 5.4B.

#### 5.4.3.2 *Body weight*

At baseline, body weight was  $16.7 \pm 0.43$  g and  $16.8 \pm 0.42$  g in the laboratory diet and mealworm fed groups respectively, ( $P = 0.89$ ). Overall, a significant difference in body weight was observed between the laboratory diet and mealworm groups (diet  $F_{1,49} = 17.6$ ,  $P = 0.003$ , diet  $\times$  day  $F_{41,419} = 3.8$ ,  $P < 0.0001$ ). During the first 21 days of the experiment, body weight increased by a maximum of approximately 13% in response to a diet of mealworms; *day 21* body weights were  $15.5 \pm 0.40$  g and  $19.3 \pm 0.44$  g in the laboratory diet and mealworm fed groups respectively (diet  $F_{1,263} = 17.84$ ,  $P = 0.002$ , diet  $\times$  day  $F_{21,263} = 10.78$ ,  $P < 0.0001$ ), Fig 5.4C. After *day 21* however, body weight in the mealworm fed group decreased and returned to baseline.

#### 5.4.3.3 *Tail width*

At baseline, tail widths were  $3.78 \pm 0.14$  mm and  $4.01 \pm 0.36$  mm in the laboratory diet and mealworm fed groups respectively ( $P = 0.61$ ). Tail width increased in response to a diet of mealworms and remained elevated above baseline for the duration of the experiment (diet  $F_{1,229} = 5.32$ ,  $P = 0.04$ , diet  $\times$  day  $F_{23,239} = 8.62$ ,  $P < 0.0001$ ), reaching a maximum width of  $4.90 \pm 0.4$  mm on *day 17* (an increase of 18%), Fig 5.4D.

| Measured parameter                                  | Mean Value     | <i>n</i> |
|-----------------------------------------------------|----------------|----------|
| Tail width (mm)                                     | 4.32 ± 0.091   | 72       |
| Body weight (g)                                     | 16.27 ± 0.22   | 72       |
| Tail weight (mg)                                    | 635.67 ± 28.39 | 43       |
| Tail triglyceride (mg)                              | 125.07 ± 8.55  | 42       |
| Body triglyceride (mg)                              | 410.07 ± 27.26 | 12       |
| Total triglyceride (mg)                             | 523.37 ± 32.05 | 12       |
| Body sub-cutaneous adipose tissue mass (- tail) (g) | 0.670 ± 0.117  | 31       |
| Blood glucose (mmol/l)                              | 3.99 ± 0.09    | 49       |
| Plasma free fatty acids (mEq/l)                     | 0.433 ± 0.076  | 27       |

**Table 5.1:** Mean values ± SE obtained for various parameters of male *S. crassicaudata* housed under *ad libitum* fed laboratory conditions.

| Parameter 1     | Parameter 2                  | r value | P value    | n  |
|-----------------|------------------------------|---------|------------|----|
| Tail Width (mm) | Body weight (g)              | 0.70    | < 0.0001 ‡ | 72 |
|                 | Tail weight (mg)             | 0.90    | < 0.0001 ‡ | 43 |
|                 | Tail triglyceride (mg)       | 0.78    | < 0.0001 ‡ | 42 |
|                 | Body fat weight (-tail), (g) | 0.78    | < 0.0001 ‡ | 31 |
|                 | Body triglyceride (mg)       | 0.43    | 0.16       | 12 |
|                 | Total triglyceride (mg)      | 0.58    | 0.05 *     | 12 |
|                 | Blood glucose (mmol/l)       | 0.14    | 0.33       | 49 |
|                 | Plasma FFA (mEq/l)           | 0.11    | 0.58       | 27 |
| Body Weight (g) | Tail triglyceride (mg)       | 0.46    | 0.002 †    | 42 |
|                 | Body fat weight (-tail), (g) | 0.87    | < 0.0001 ‡ | 31 |
|                 | Body triglyceride (mg)       | 0.37    | 0.07       | 24 |
|                 | Total triglyceride (mg)      | 0.46    | 0.02 *     | 24 |
|                 | Blood glucose (mmol/l)       | 0.12    | 0.42       | 49 |
|                 | Plasma FFA (mEq/l)           | 0.41    | 0.03 *     | 27 |

**Table 5.2:** The relationship between tail width (mm), body weight (g) and a range of other parameters in LD male *S. crassicaudata* ( $n = 12-72$ ). \*  $P \leq 0.05$ , †  $P < 0.01$ , ‡  $P < 0.0001$ .

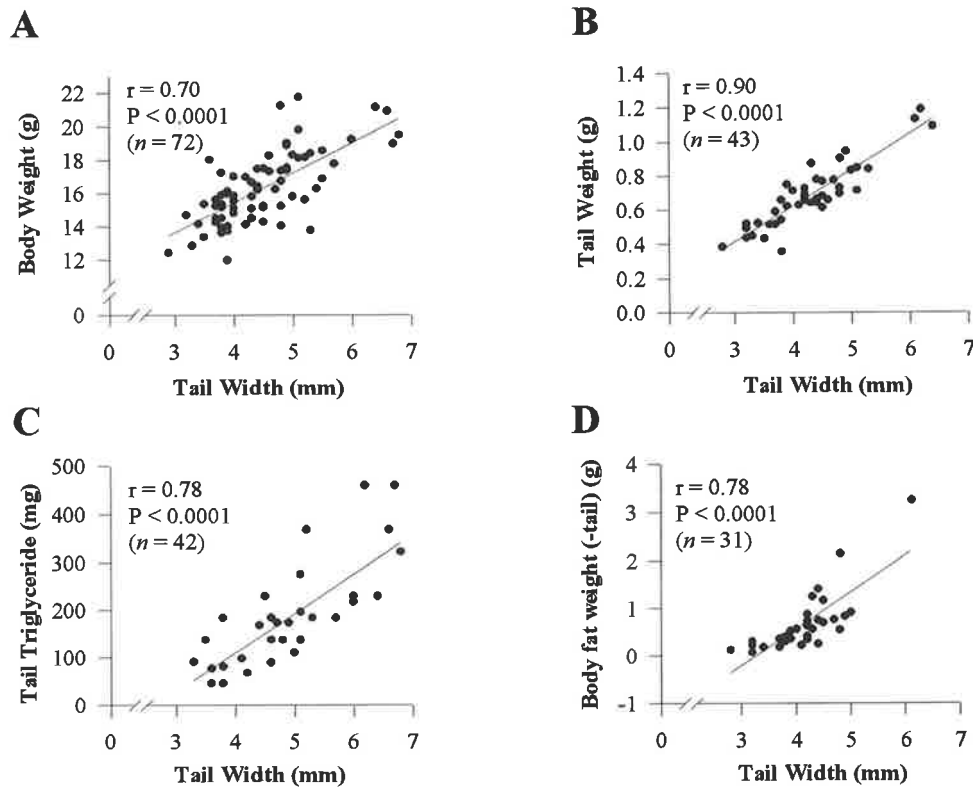


**Figure 5.1:** Tail width (mm) of *S. crassicaudata* is positively correlated with body weight (g; A), tail weight (g; B), tail triglyceride content (mg; C), and body fat weight (minus the tail fat) (g; D).

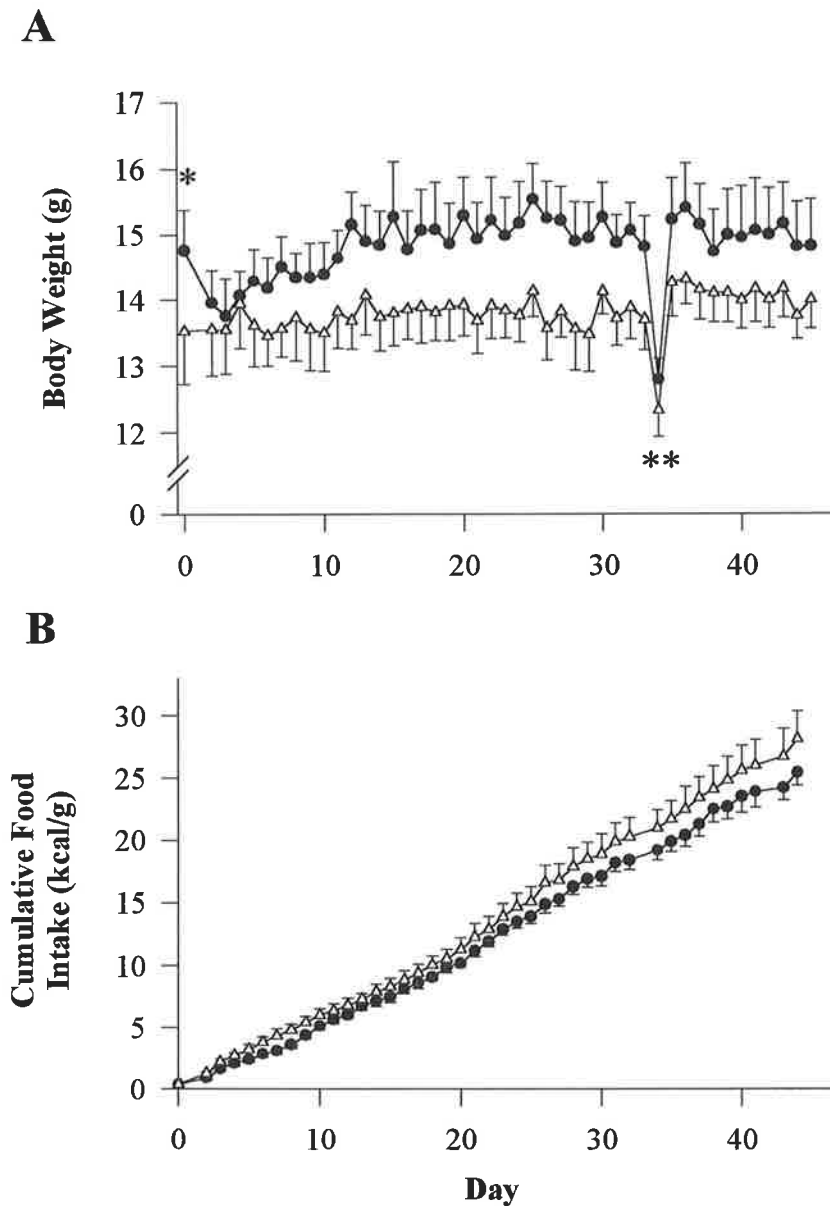


**Figure 5.2:** Daily body weights (g, A) and cumulative food intake (kcal/g body weight, B) of LD male *S. crassicaudata* with ( $\Delta$ ) and without ( $\bullet$ ) tails. A period of 24-hour food deprivation occurred on day 33. Body weight decreased following lipectomy, however body weight subsequently returned to baseline. 24-hour fasting induced a decrease in body weight in both groups. No significant difference in energy intake was observed. \*  $P < 0.05$ ; \*\*  $P < 0.005$ . Values are mean  $\pm$  SE.



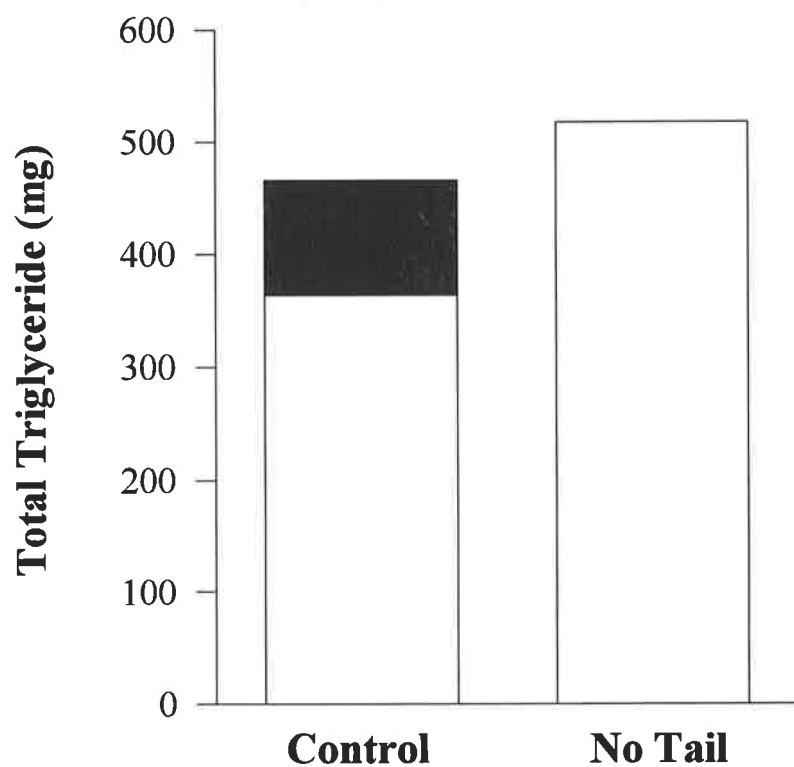


**Figure 5.3:** Mean triglyceride content (mg) of the tail (closed bar) and body (open bar) in LD male *S. crassicaudata* with and without tails (45 days after tail removal). At sacrifice, total triglyceride content of the control animals was not different from the ‘no tail’ animals.

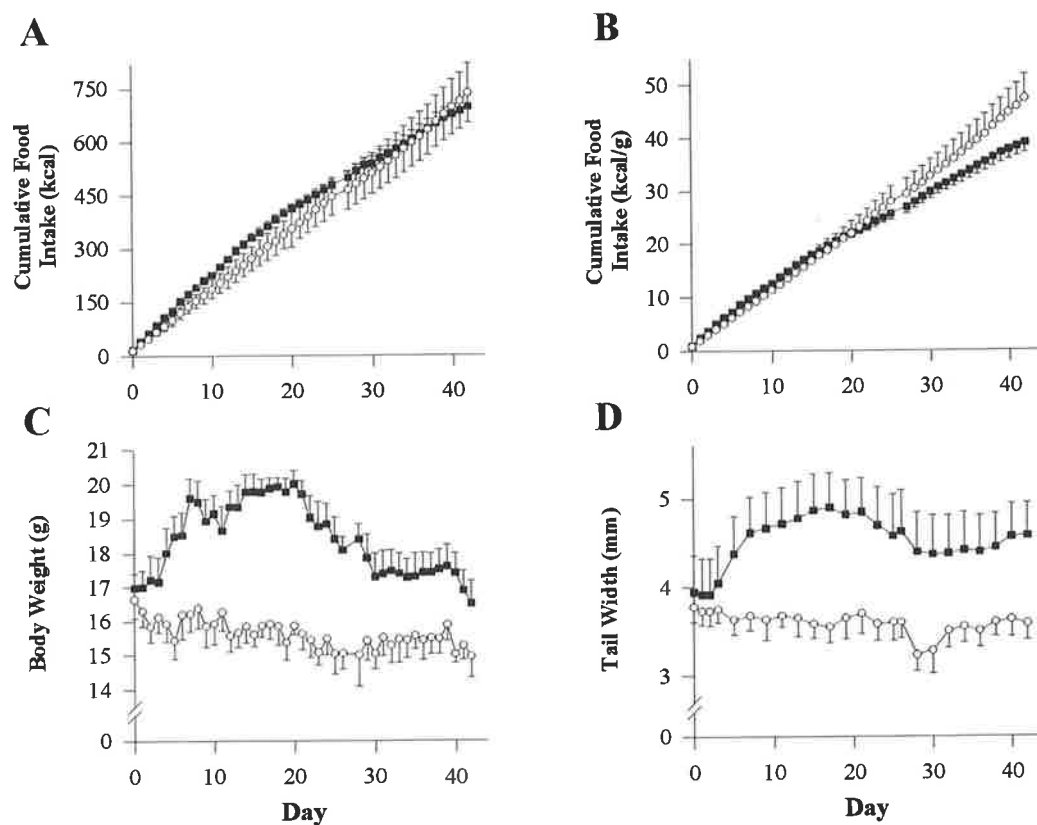


**Figure 5.4:** Mean  $\pm$  SE cumulative food intake in kilocalories (A) and in kilocalories per gram of body weight (B), body weight (g; C), and tail width (mm; D) of long-day housed (16-h light, 8-h dark) *S. crassicaudata* given either standard laboratory diet (○) or mealworms (■) *ad libitum* for 42 days. Before commencement of the study, animals had constant access to standard laboratory diet only.

## 5.5 DISCUSSION

The results of these studies demonstrate that both male and female *S. crassicaudata*, store approximately 25% of adipose tissue in the tail as triglyceride. Tail width is a reliable and accurate measure of changes of not only tail fat, but also overall adiposity. Following tail removal (~ 25% lipectomy), fat was regained subcutaneously. A high calorie, increased fat diet induced an increase in adiposity, characterised by a sustained increase in tail width. From these data it can be concluded that while fat stores are rigorously defended below a 'set-point', the upper limit for fat storage is more flexible and there is a broader range of tolerance, although a plateau is reached, presumably in accordance with genetic potential (West *et al* 1995).

In *S. crassicaudata*, the tail accounts for ~ 4% of total body weight, and the tail triglyceride store accounts for ~ 1% of total body weight. The triglyceride content of fat has been estimated to represent 60-80% of total fat (Christie 1982, Gurr & Harwood 1991). Since tail fat of *S. crassicaudata* accounts for such a small proportion of total body weight, changes in tail fat may not necessarily be reflected by alterations in body weight. In addition, *S. crassicaudata* is extremely lean; body triglyceride stores contribute to a small proportion (~ 2.5%) of total body weight. Measurements of tail width are therefore more likely to represent changes in overall adiposity than measurement of body weight.

The observation that fat stores were regenerated following lipectomy, without a significant increase in energy intake, suggests that the additional energy required to

increase the remaining body fat stores occurred primarily as a result of either a decrease in energy expenditure or an increase in metabolic efficiency. These findings are consistent with the reported effects of lipectomy in Syrian hamsters (Halaas *et al* 1995), Siberian hamsters (Mauer & Bartness 1994), and ground squirrels (Dark *et al* 1985). In other rodents, the effects of lipectomy are more variable and depend on the age of the animal, the strain (animals with genetic or acquired obesity syndrome show a more rapid and complete response), gonadal function, and dietary fat content (Weigle 1994). The failure to demonstrate an increase in food intake in many previous lipectomy studies has been attributed to post-surgical effects including inflammation, or alterations in the production of gonadal hormones (Chlouverakis & Hojincki 1974, Faust *et al* 1984, Faust *et al* 1977a, Liebelt *et al* 1965). In contrast to the relatively dramatic surgical procedures required to achieve lipectomy in rodent experiments, tail removal in *S. crassicaudata* proved to be a minor procedure. The data is consistent with the physiological regulation of total fat mass, but does not support the concept that levels of body fat are tightly coupled to regulation of food intake. The restoration of fat mass after moderate lipectomy is accounted for predominantly by reduced energy expenditure or increased metabolic efficiency and further studies are required to determine the mechanisms involved.

In *S. crassicaudata*, both body weight and fat mass (tail width) increased when fed a highly palatable, preferred diet of mealworms, which are calorie dense and have a greater fat and protein content than standard laboratory diet. Body weight, but not tail fat stores, subsequently returned to baseline, suggesting an overall increase in fat storage. The diet of mealworms resulted in a maximum increase in body weight and

tail width of 13% and a 17.7% respectively. In rats, an increase in dietary fat and calories results in increased fat stores under *ad libitum* feeding conditions (Salmon & Flatt 1985). For example, in rats fed isocaloric diets varying in fat content (12, 24, 36, 48%), total body fat and adipose depot weights increased in proportion to the level of fat in the diet. Furthermore, the increase in body fat occurred in the absence of significant changes in body weight (Boozer *et al* 1995). *S. crassicaudata* that received the mealworm diet consumed 10% more calories and 39% more calories as fat than the animals receiving standard laboratory diet. Small daily increases in energy intake may have been sufficient to predispose *S. crassicaudata* to an increase in adiposity. There is evidence, however, that an increased intake of fat alone may have increased adiposity. During the course of this research, our group has shown that as in rodents (Boozer *et al* 1995, Salmon & Flatt 1985) and humans (Dreon *et al* 1988), body fat stores in *S. crassicaudata* increase in response to increased dietary fat (10, 20, 40%), independent of total calories (Ng *et al* 1999).

The simplicity of the lipectomy procedure, and the observation that fat stores are regenerated, characterises *S. crassicaudata* as a usefull animal model in which to investigate the regulation of body fat mass. This model will also be usefull to investigate the mechanism whereby animals vigorously resist a decrease in body fat mass below a 'set-point', but at the same time allow plasticity of the upper limit.

## CHAPTER 6

**CLONING OF LEPTIN cDNA AND ASSIGNMENT TO THE  
LONG ARM OF CHROMOSOME 5 IN *S. CRASSICAUDATA*****6.1 SUMMARY**

Examinations of leptin and its molecular characteristics have demonstrated that the *ob* gene coding sequence shows high levels of evolutionary conservation amongst vertebrate species. To date, however, the leptin sequence from a marsupial has not been determined. In this study, full-length cDNA clones for *S. crassicaudata* leptin were isolated and sequenced. Southern and *in situ* hybridisation data indicated a single leptin gene in the *S. crassicaudata* genome, localised to arbitrary chromosome bands 5q24-q31 on the long arm of chromosome 5, the short arm terminus of which bears the only nucleolar-organising region. The nucleotide sequence of the cDNAs revealed that the primary translation product of *S. crassicaudata* leptin is composed of 167 amino acid residues with a potential signal peptide of 21 residues. The mature protein of 146 amino acids is 82% similar to both the mouse and human proteins, and is predicted to have a molecular weight of 16.26 kDa. Northern blot analysis revealed that the corresponding mRNA is approximately 3.9 kb in size and is expressed only in

white adipose tissue of this marsupial species. Evolutionary analyses indicate that *S. crassicaudata* leptin cDNA has evolved at a significantly faster rate than cDNAs from eutherian mammals.

## 6.2 INTRODUCTION

*Sminthopsis crassicaudata* regulates its body fat stores; studies presented in this thesis have demonstrated that *S. crassicaudata* store ~25% of adipose tissue in the tail, and after removal of the tail, fat re-accumulates in sub-cutaneous adipose tissue depots (see chapter 5). This re-accumulation of fat occurs without any significant alterations in energy intake, implying modifications in energy expenditure. The defence of fat stores observed in *S. crassicaudata* is consistent with that seen in many other mammals (Harris 1990, Leibel *et al* 1995), and supports the lipostatic hypothesis postulated by Kennedy (1953), that the size of fat depots is sensed by the central nervous system through a product of fat metabolism that circulates in the blood, and interacts with the hypothalamus to affect energy balance. The parabiosis experiments of Coleman (1973) provided convincing evidence for a circulating satiety factor (see section 1.3.1.2) and, in 1994, the *ob* gene was cloned from the *ob/ob* obese mouse (Zhang *et al* 1994).

Leptin, the 16 kD protein product of the *ob* gene is produced by adipocytes and is expressed predominantly but not exclusively in white adipose tissue. Leptin circulates in the plasma in proportion to total adipose tissue stores (Considine *et al* 1996,

Maffei *et al* 1995), and levels of expression of the *ob* gene correlate with body fat content (Frederich *et al* 1995b, Maffei *et al* 1995). Leptin thus provides a communication link between fat tissue and the brain, acting on receptors found primarily in hypothalamic nuclei (including the arcuate nucleus, ventromedial hypothalamus, lateral hypothalamus and dorso-medial hypothalamus) (Mercer *et al* 1996, Schwartz *et al* 1996b). Leptin partly controls body fat stores through co-ordinated regulation of feeding behaviour, metabolism, neuroendocrine responses, the autonomic nervous system and energy balance. Both central and peripheral leptin administration reduce adipose tissue stores (Campfield *et al* 1996, 1997). The genetically obese *ob/ob* mouse does not produce active leptin due to a nonsense mutation at codon 105 (Zhang *et al* 1994). Leptin replacement corrects the metabolic abnormalities of the *ob/ob* mouse that include hyperphagia, hyperglycaemia, hypothermia and impaired reproductive function (Campfield *et al* 1996, 1997, Halaas *et al* 1995, Pellymouter *et al* 1995, Weigle *et al* 1995). The functions of leptin and the *ob* gene are discussed further in section 1.5 and in chapter 7.

Leptin has now been cloned and sequenced from a number of eutherian mammals, including the human (Gong *et al* 1996), rhesus monkey (Hotta *et al* 1996), mouse (Zhang *et al* 1994), pig (Mendiola *et al* 1997), rat (Murakami & Shima 1995), and sheep (Dyer *et al* 1997). Leptin has also been cloned from the chicken (Taouis *et al* 1998) and turkey (Ashwell *et al* 1998). To date, there is no information, at the molecular level, on leptin from any marsupial species. Studies on marsupial leptin are of interest in an evolutionary context because of the early separation of the marsupial and eutherian lineages, estimated to have occurred ~ 150 million years ago (Hope



RM *et al* 1990, Kirsch 1984, Kumar & Hedges 1998). The aim of this study was, therefore, to isolate and sequence the cDNA for leptin from adipose tissue of *S. crassicaudata*, to establish the chromosomal localisation of the leptin gene, and to investigate the tissue distribution of leptin mRNA.

## 6.3 MATERIALS AND METHODS

### 6.3.1 *Cloning of S. crassicaudata leptin*

Total RNA was isolated from *S. crassicaudata* white adipose tissue using the method outlined in section 3.8.1. First strand cDNA was synthesised using RNase H-Superscript reverse transcriptase (Gibco BRL, MD, USA). Second strand cDNA synthesis was carried out according to the method of Gubler and Hoffman (1983). Following ligation of the cDNA to EcoRI adaptors (Pharmacia, NJ, USA), the cDNA was ligated to EcoRI digested  $\lambda$ ZAPII vector (Stratagene, CA, USA). The library was screened with a mixed  $^{32}\text{P}$  labelled probe comprising the coding sequence of murine and human leptin cDNA. The filters were hybridised with the labelled probe overnight at 62°C in a hybridisation solution containing 5 X SSC, 1mM EDTA, and 0.1% SDS. The filters were washed at 55°C in 1 X SSC, 1mM EDTA and 0.1% SDS. Positive clones were isolated and plaque purified. Phagmids were isolated from the positive phage by *in vivo* excision and the cDNA insert sequenced. The sequence of the leptin coding region was derived from four independent clones, named pSLMFL1, pSLMFL2, pSLMFL3, and pSLMFL4.

### 6.3.2 *Leptin sequence analysis*

Leptin cDNA and protein sequences were extracted from GenBank. Species names and locus identifications are: Human, *Homo sapiens* (G1226244); Gorilla, *Gorilla gorilla* (G1620743); Orangutan, *Pongo pygmaeus* (G1620745); Rhesus monkey, *Macaca mulatta* (G1389762); Pig, *Sus scrofa* (G1488299); Ovine, *Ovis aries* (G1813869); Bovine, *Bos taurus* (G1167990); Dog, *Canis familiaris* (G4009340); Rat, *Rattus rattus* (G995615); Laboratory mouse, *Mus musculus* (G603288); Turkey, *Meleagris gallopavo* (G3435284); and Chicken, *Gallus gallus* (G2406650). Sequence alignments were carried out using the algorithm CLUSTAL-W (Thompson *et al* 1994), and were edited and displayed using the computer program GeneDoc (Nicholas & Nicholas Jr 1997). Phylogenetic trees were derived from the aligned sequences using DNAPARS (DNA maximum parsimony), DNAML (DNA maximum likelihood), PROTPARS (protein maximum parsimony). Bootstrap analyses were carried out using SEQBOOT and majority rule consensus trees were derived using CONSENSE. These algorithms were from PHYLIP (Phylogeny Interface Package), (Felsenstein 1993). Relative rates tests (Wu & Li 1985) were carried out using the algorithm K2WuLi (Jermiin 1996).

### 6.3.3 *Southern and Northern blotting*

#### 6.3.3.1 *Leptin cDNA probe*

A ~1.8 kb *S. crassicaudata* leptin cDNA fragment from clone pSLMFL2 was used to hybridise the genomic and RNA blots. The probe was labelled with [ $\alpha$ -<sup>32</sup>P]dCTP (Bresatec, SA, Australia) to a specific radioactivity of approximately  $1 \times 10^9$  dpm/ $\mu$ g DNA using a Gigaprime DNA labelling kit (Bresatec, SA, Australia).

### 6.3.3.2 *Southern blotting*

Fifteen micrograms of *S. crassicaudata* genomic DNA was digested overnight with EcoR1, and the products electrophoresed on a 0.8% agarose gel, together with  $1 \times 10^{-5}$   $\mu$ g of the undigested pSLMFL2 plasmid clone. DNA was visualised by staining with ethidium bromide and then transferred overnight to Hybond-N<sup>+</sup> nylon transfer membrane (Amersham Pharmacia Biotech, NSW, Australia) by capillary blotting. Pre-hybridisation was performed for 4 hours at 65°C in 5 X SSPE/0.5% SDS + 5 X Denhardt's, with 100  $\mu$ g/ml of denatured salmon sperm DNA. Genomic Southern blots were hybridised with the cDNA probe at  $1 \times 10^6$  cpm/ml, for 18-24 hours at 65°C. Post-hybridisation washes consisted of 2 x 10 minute washes at room temperature in 2 X SSC/0.1% SDS and 2 x 30 minute washes at 65°C in 1 X SSC/0.1% SDS. Blots were then exposed to Kodak X-omat AR film (Integrated Sciences, NSW, Australia) for 7 days at - 80°C.

### 6.3.3.3 *Northern blotting*

Total RNA was extracted from *S. crassicaudata* white adipose tissue (WAT), heart, kidney, spleen, bowel, liver, skeletal muscle, interscapular adipose tissue and cortex, and from mouse WAT and brown adipose tissue (BAT), as above. Agarose gel electrophoresis and Northern blotting were performed as described in sections 3.8.1 and 3.8.3. The pSLMFL2 cDNA probe was used at  $1.25 \times 10^6$  counts/ml. Blots were exposed for 4 days at -80°C.

#### 6.3.4 *Chromosomal localisation of S. crassicaudata leptin cDNA*

Chromosomes were derived from splenic lymphocytes cultured in AmnioMax (Gibco-BRL, Life Technologies, Vic, Australia) and stimulated with 5 µg/ml concanavalin A (Sigma Chemical Company, NSW, Australia). After 3 days culture, 5-bromodeoxyuridine (20 µg/ml) was added to the culture medium for the final 7 hours, followed by 1 hour of spindle fibre disruption with colchicine. After initial failure of fluorescent *in situ* hybridisation (FISH), radioactive *in situ* hybridisation was applied as described by Webb (1999). Specifically, the 1.8kb pSLMFL2 leptin cDNA probe (described above) was labelled by nick translation with tritium to a specific activity of  $5.6 \times 10^7$  cpm/µg and applied to the slides at a concentration of 400 ng/ml, in the presence of 400 µg/ml of sonicated salmon sperm carrier DNA. Slides were then exposed to 1:1 diluted L4 emulsion (Ilford, SA, Australia) for 42 days. Localisation by FISH was successfully repeated using the 1.8 kb pSLMFL2 cDNA probe and a 5 kb cDNA of the 18S ribosomal RNA gene of the rat (Katz *et al* 1983) as co-hybridised probes. The 18S probe was specific for the single nucleolar-organising region (NOR), which has been previously shown to be on the short arm of chromosome 5 in *S. crassicaudata* (G Webb, personal communication). Both probes were labelled using a BioNick kit (Gibco-BRL, Life Technologies, Vic, Australia); the leptin probe incorporated 111.5 pg and the 18S probe 276.5 pg of biotin per µg of DNA. Subsequent hybridisation immunochemistry and staining for R-bands (Lemieux *et al* 1992) was performed as previously described (Webb *et al* 1997).

## 6.4 RESULTS

### 6.4.1 *Isolation and sequence of S. crassicaudata leptin cDNA*

Phagmids were isolated from positive LZap II phage by *in vivo* excision. The cDNA inserts of two clones, pSLMFL2 and pSLMFL4 were analysed in detail. The sequence of pSLMFL4 is given in figure 6.1. Both clones were found to contain an identical open reading frame of 501 nucleotides which is flanked by initiation and termination codons. The two clones differ in the length of their putative 5' and 3' untranslated regions (UTR). The putative 3' UTR of clones pSLMFL2 and pSLMFL4 are approximately 1.2 and 3.4 kb in length respectively, and the putative 5' UTRs are 53 and 283 nucleotides respectively. The first 19 nucleotides of clone pSLMFL2 are not identical to the corresponding sequence of pSLMFL4. The junction where the two sequences deviate contains a splice acceptor sequence on pSLMFL4 and is situated in a relative position where intron 1 interrupts the mouse and human leptin genes (He *et al* 1995, Isse *et al* 1995). It would appear that pSLMFL4 may represent a cDNA of a partially processed leptin transcript in which intron 1 is left un-spliced.

The cDNA clones were predicted to encode a primary translation product of 167 amino acids. This protein contains a putative N-terminal signal sequence of 21 residues. The mature protein contains 2 cysteine residues (positions 117 and 167) and has a predicted molecular weight of 16.26 kDa, with an isoelectric point of 6.09.

#### 6.4.2 *Comparative analysis of S. crassicaudata leptin protein*

Amino acid sequence alignments, Fig 6.2, show that leptin is highly conserved amongst mammals. For the mature leptin peptide, sequence similarities (sequence identities in brackets) between *S. crassicaudata* and selected mammals are: human 82% (70%), mouse 82% (69%), rat 82% (69%), and pig 84% (73%). This level of conservation extends to two avian species where the sequence similarities (identities in brackets) with *S. crassicaudata* are: turkey 79% (66%), and chicken 80% (67%). The signal peptide region is less well conserved between species. Two cysteine residues are present at identical sites (positions 117 and 167) in the mature peptide of all species.

#### 6.4.3 *Phylogenetic analyses*

Maximum likelihood analysis of the aligned cDNA sequences encoding the mature leptin protein produced the phylogenetic tree shown in Fig. 6.3. When maximum parsimony and bootstrap analyses were carried out, a consensus phylogenetic tree of the same general topology was derived (results not shown), with bootstrap values that were all less than 75%, many being less than 60%.

#### 6.4.4 *Expression of S. crassicaudata leptin*

Southern blot analysis of genomic DNA using the 1.8 kb *S. crassicaudata* leptin cDNA clone pSLMFL2 as a probe resulted in a single hybridisation band of approximately 10 kb. A single band of ~ 5.2 kb was detected for the undigested pSLMFL2 plasmid, Fig 6.4.

Northern blot analysis revealed the expression of leptin mRNA, as a single 3.9 kd band, in *S. crassicaudata* WAT, but not in BAT, cortex, muscle, liver, bowel, spleen, kidney or heart. The probe did not cross hybridise with mouse RNA from either WAT or BAT, Fig 6.5.

#### 6.4.5 *Chromosomal localisation of S. crassicaudata leptin cDNA*

Chromosome 5 in *S. crassicaudata* is obvious from its size and arm ratio, but R-banding produced by late-S labelling with 5-BrdU was also used, Fig 6.6. Initial scoring of signals from tritium, 268 silver grains, to all chromosomes of a female *S. crassicaudata*, revealed a major peak of silver grains, containing 43.3% of the grains, over the long (5q) arm of chromosome 5. A tallest column, of 26 grains, was over the interface of faint and dark R-bands, situated at approximately 67% of the length of the arm distal to the centromere (data not illustrated). By contrast, the average labelling intensity over the whole karyotype was only 2.33 grains. Background was limited to a maximum of 3 grains with the exception of chromosome 6, which was labelled at an average labelling intensity of 3.40 grains, with tallest columns, of 5 grains, over each arm.

A repeated analysis with tritium was done for the critical 5q arm, using only well-banded chromosomes and scoring onto an R-banded idiogram constructed largely from the observed bands, which were arbitrarily numbered, Figs 6.6a,b. This scoring showed a repetition of the peak of grains to arm 5q, with the two tallest columns over the interface of faint and dark R-bands, Fig 6.6a. The location of the gene is regarded as being near the interface of the bands which are arbitrarily numbered q2 and q3.

More specifically, the position of the four tallest columns containing 56% of the grains over the arm 5q, in the sub-bands 5q24-q31, Fig 6.6a.

Co-hybridisation of the biotinylated leptin and 18S probes showed labelling in the vicinity of the leptin locus on 31.5% of 200 NOR-bearing chromosomes 5. Leptin fluorescein isothiocyanate (FITC) signals, Fig 6.6d, photographed on colour-positive film, were located on an R-banded idiogram of chromosome 5, as previously, Fig 6.6c. The signals were more spread out than expected for FISH, probably due to the difficulties of banding a small chromosome. The result for FISH was similar to that for tritium, but with slightly more signals scored over the faint band q2, Fig 6.6c, resulting in a probable localisation to sub-bands 5q23 on the proximal half of q31. In a few cases the NOR was seen to be not quite at the terminus of the short arm of chromosome 5, Fig 6.6d (top). The signal to background ratio obtained with FISH was very similar to that obtained with tritium labelling. On the photographs, which recorded 139 leptin FITC signals, Fig 6.6c, there were 119 background signals of similar size and 21.8 % of the background grains were over chromosome 6. This confirms that chromosome 6 is over-labelled by comparison with the chromosomes other than 5, since chromosome 6 has only approximately 9.4 % of the length of the non-target chromosomes.



**Figure 6.1:** Nucleotide and derived amino acid sequences of *S. crassicaudata* leptin cDNA. Nucleotides are numbered from the first base determined at the 5' untranslated end. The 167 amino acids are numbered from the start methionine. Initiation (ATG) and termination (TGA) codons are shown in bold, and the putative signal peptide sequence is underlined. Two cysteine residues, at positions 117 and 167, are in bold and underlined.

|      |                                                                          |      |
|------|--------------------------------------------------------------------------|------|
| 1    | GGCACGAGGCGCTGGGAAGCAGGGGGGAGTGAGTGCTCAGCCAGCCCTGGAGGAGCCGAGATCTTGAACC   | 70   |
| 71   | TCCACATTGGAAAGCCCCCTGGACAAGCGGTGATCCATGGAGGGGAATTTCTAGATCTGTCCAAGGAGAAGG | 140  |
| 141  | TCCCTATTTTCAGAGCTGAGATGCTTTTGAAATTACCCAGAGCACATCTCCAAGAACTACGGATTCTCCTT  | 210  |
| 211  | CCAGAGAGGACAGTGAATTTTCGCTCAAGCTTGGAAAGCTGAGGTGAGACAAGCACATTCACCTTGGAGAGG | 280  |
| 281  | AAAATGCAATTGCGTGCCTTTTGTCTGCTTCTTGTGGTTTTGCCACCACCTCTACTACAGCCAAGCGGTAC  | 350  |
| 94   | <u>M H C V P L F C F L W F C H H L Y Y S Q A V P</u>                     | 117  |
| 351  | CCATCCGAAAAGTCCAAGATGACACAAAAACCTCACCAAGACCATCATCACGAGGATCAATGACATCTC    | 420  |
| 117  | I R K V Q D D T K T L T K T I I T R I N D I S                            | 140  |
| 421  | GCACATGTACTCAATTTCTGCCAAACAGAGGGTAAGTGGCTTGGACTTCATCCCAGGACTCCATCCCTTC   | 490  |
| 141  | H M Y S I S A K Q R V T G L D F I P G L H P F                            | 164  |
| 491  | CAGAGTCTGTGACACATGGACCAGACTTTGGCCATCTACCAGCAGATCCTCTCCAATCTATCTTCCAGAA   | 560  |
| 164  | Q S L S D M D Q T L A I Y Q Q I L S N L S S R N                          | 187  |
| 561  | ACATGGTGCAAATATCCAATGACCTTGAGAACCTCAGAGACCTTCTCCATTTGCTGGGTTCTTAAAGAG    | 630  |
| 187  | M V Q I S N D L E N L R D L L H L L G S L K S                            | 210  |
| 631  | CTGTCCCTTCGATGAAGCTGGTGGTTTGAGTGCTTTGGGGAATTTGGAAGGTGTGATGGAAGCCTCTCTC   | 700  |
| 211  | <u>C</u> P F D E A G G L S A L G N L E G V M E A S L                     | 234  |
| 701  | TATTCACGGAAGTGGTGACCTGACCCGACTTCAGAAGTCCCTATATGTGTCATGCTCCAACAGCTGGATC   | 770  |
| 234  | Y S T E V V T L T R L Q K S L Y V M L Q Q L D L                          | 257  |
| 771  | TCATCCATGTTGCTGAAGCCAGAGACAGAGACAGAGAGAGAAAAAGAGAGAGGGGGGAGTTTCTTTCC     | 840  |
| 257  | I H G <u>C</u> *                                                         | 280  |
| 841  | CCTGGTTCATTAAAAAACCTAGAGTAGAAAACCTCTTTATTCTTCAGCAAAATCTAGACTTTTGACTGTA   | 910  |
| 911  | TCCTTTACCTAGGATATAGGATCACTAAGTACCCTATGAATCCTCCAGTCATATCTTTCAAGGCAAAAC    | 980  |
| 981  | AAAATGTCATATACAGCTGGAGACTAAAGACCTACACATATACCAAAATTTGGCTCAAGTTGCCGTTCAA   | 1050 |
| 1051 | TGAACAGTGAGTGGGTACATCACAGTGATGTTCTTATTGAACCTTCAGTACTGACAGAGCTGATATCT     | 1120 |
| 1121 | ATCCCTTCTTACCTCCTTGCCAAACCTCTCGCCTTCTTCCCCAATCCCCCTACTACACATTAATGTG      | 1190 |
| 1191 | AGCAGTCGTGACTTCGGGCAATTGGAACATTAATTTTTCCTGGTAACTTCGAAGAGTCATCAGAAGATT    | 1260 |
| 1261 | TCAATTTGAAATGACACTTGTGGGGAGCCCCATTTGAATTATTAAGAAATGGCCCCCTAGATGCAGTTGG   | 1330 |
| 1331 | CAACTCCTAAGTGATACGACTTAGAACTTCTATTATTGTTGGGTTTTGGTTTTTGTTTTTTGTGTGGT     | 1400 |
| 1401 | TGTTTTTGTGTCATTTACTTTTATGGTCTATTCCAGACCAGCTCAGACTGAGTGCTTCAGAATCAGTACT   | 1470 |
| 1471 | GCTTTTGGAGATGTAAGACTGGGTCTGTGAGTGACTTTGGGAACATCGGGGCTTTCAGCCAGACCC       | 1540 |
| 1541 | AGCCAAAGCAAGTGAATTAACCTCTTCTCACTCTTTATTCTTCTGGAAGTGAATTAAGTGA            | 1610 |
| 1611 | TATATTTTCATTGGCTTCTATGCCATTTCAAGTGGATCACTAAGGACCAGATTATTTAAGGGGAAAAAA    | 1680 |
| 1681 | TTTGAGAATTGCTTTGAAATGGAATGTTGAGCAATTCAGGGTAGGATGGGATGGGATTACATTTCCAG     | 1750 |
| 1751 | GGTAAAAATGGGTTGTTACTTTGTGTGTCCTTTGATAACATTTAGCATAAAGAATCCTTTCCCCACTTCT   | 1820 |
| 1821 | CCTTTTCACTCTCTACTACTCACTCAAGTATGAGAACCCATGAGGATGGGTTGCTACTAAAAGTACTG     | 1890 |
| 1891 | CAATGCCAATTTCTCAGTTCTCATAGCCACCCAGAAGAATCTTCCCCCCCCAAAAAAGAACAGCA        | 1960 |
| 1961 | AAAGGAAATCAGGGAGAAGAGAAAACAGGTAGGAAGCGACAAATCATAGTCAAGCTATCTCGAAGAACTG   | 2030 |
| 2031 | TAGAGCAATTTCAAGGAATTTGTGAAATTTGGGGCTTTGAGACTCCGAACAGCTGTTTGCTAATGGCAGT   | 2100 |
| 2101 | CGTAAACTTGGGGTTAGGAACAGAGTGAGAATGCAAGAGCACTGAAGGCAGAGACAACCCCTTAGGAAA    | 2170 |
| 2171 | GTATTTCTAGATGGGGGCTGGGAGAGTGGAATGGAGAATGGCAACCAAGAAATGTACACACACT         | 2240 |
| 2241 | CACCTCTGACTGAGGAAATGAAAGCAATAAATCCCCAAGAGAGAAGGAAACAGACTCATTTGGCTAATT    | 2310 |
| 2311 | GTGATGGACTCTCTCCCTTTTCTCTAACAACAAAAGACCTTACAAGTCACATAAGGGAAGAGAGAAAGAA   | 2380 |
| 2381 | ATAAGCATTATTTAGTTTACTCTGTGCCAGGCAGTGTGCTAAGTACTTTATAATTATCTCATTTGATAT    | 2450 |
| 2451 | AATCTCCATTTTATCTCATTAGACAAAACCTCTCCGGGCCTCAGTTTTCTCATCTGTAAAATGAAAGGAT   | 2520 |
| 2521 | TAGTTACAATCTCTCCAGCTCTAAATCTGTTTTCCTATGAGTATGCTTGTCTTTCTCCATCACCATC      | 2590 |
| 2591 | CAGCGTTTATGTGGAAGAAAGCTTCTACATATTTAATGGAGACTCTGAACCTCAAACAGTGTTTCTTA     | 2660 |
| 2661 | CCTAGAGCGCTCTGGTTCTCCTTGCAGCCTCAGGCTAATTGTTTATTGTTTGTACAATGAAAGTGTCTGT   | 2730 |
| 2731 | ACTTGAGCCTCTGGCATGAATTAATCACTCCTTTGTGGTGTCTTTTTTCACTCTCTCCTGTTTCATTGAG   | 2800 |
| 2801 | CCGGTAGAGGCAGAGTAGGATAGTTGGAAGGTTGTGGATTGCAATTCAGAAAACCTGGGTTTAGAATCC    | 2870 |
| 2871 | AAGCTCTGTCACTTACTTGTTTGGGCAAAATCATTTAATCATTTGAACTTGAACCTCAGTCCCTCAAGGGT  | 2940 |
| 2941 | TGGACTAGTGACCTCTAAAACTTCTCAGCTCTAGTCAATGATCTAATGATAAGGATTTCTCTTTTC       | 3010 |
| 3011 | CATTGGTGAGGTTCTTGTATGAGGATCAAAAAGGACCTCGGATGGTCTCTGGTCTAAAGATCTGGATC     | 3080 |
| 3081 | GGTACTAGTGAGATATCAGAATGAAAGTGAATCGCGTCTATAAGATAAGCTACCTGTGGCCAGAGCTAT    | 3150 |
| 3151 | TGATCCAAGCCATGAGGGCACATAGGAAGGTATGAGATTTTCTCTTTAAAAATATACCAGCCTTTAAAAAG  | 3220 |
| 3221 | GCTACTTTTAGAGACTAGTTTGGGCTGTTTTTCAAGCTAAATGAAGTTGTCATGTTGCTTAGATATTG     | 3290 |
| 3291 | CTTGTGGAGGACTGAACAGCACCATATATAATATTTGTTAGTCAATGCTGACCTCTCTGAGCCCTTGA     | 3360 |
| 3361 | ACCATACTGTTTTCTTGCAAAATATCTGGTGTGGCTTGTCTATTTCCTTCTCCAAGGAAAAACAAAAGTTA  | 3430 |
| 3431 | AGTGACTTGCCCAAGTTCACATAGGAAGTTTCTGAGGTTGGATTGAACTCAGATCTTCTCAAGTCCATC    | 3500 |
| 3501 | ACTTTATCCACTGCACCACCTAAGTGCCTCTACCATATAATATAAAGAGATAGAAAAGAAAAGAGAAAAA   | 3570 |
| 3571 | ACCCATTCTTTGCTCTCAAGGAAGTGGTATCCTAGTTGGGGAAAAAAGTGAACCTTCTGCCTCTG        | 3640 |
| 3641 | GTTATAACAGAAATAAAAAGCATAAATGCAAGCAAAACATATTAGCAAGGCAATAAATAGCTGAG        | 3710 |
| 3711 | CACCAAACTCTTGGGGGAAACCAACCGGTAGTAAGTACTATCATTCTCATCTCTCCAAACCCCTTT       | 3780 |
| 3781 | CACCTTTCATTCTCACCTCCCAACAACATTTTAAAAGTAGCTCTGGCAGTTTTTTTTTATTTTGAATAATT  | 3850 |
| 3851 | TGAATTATTTCAATTTGAAAATAAGAGAGCTTAAAGTATCCAGAGGTTAAAAGATTAACTGTAATATGAGA  | 3920 |
| 3921 | TTGAATACTTAGCAAGTGGTATGGCTGGGCTCCTGATTCTTGATTTGTTGGACTGTGAGCCCGCTTAGGG   | 3990 |
| 3991 | AGACTGAACATGCTTAAGTTGTTTTGGACGAGAAGGCTTCTGGAAAGGGAAAAATTTTGAGGTAAGTTT    | 4060 |
| 4061 | GGAAAGGGCAAGCATGATGAGAATTAGTAG                                           |      |

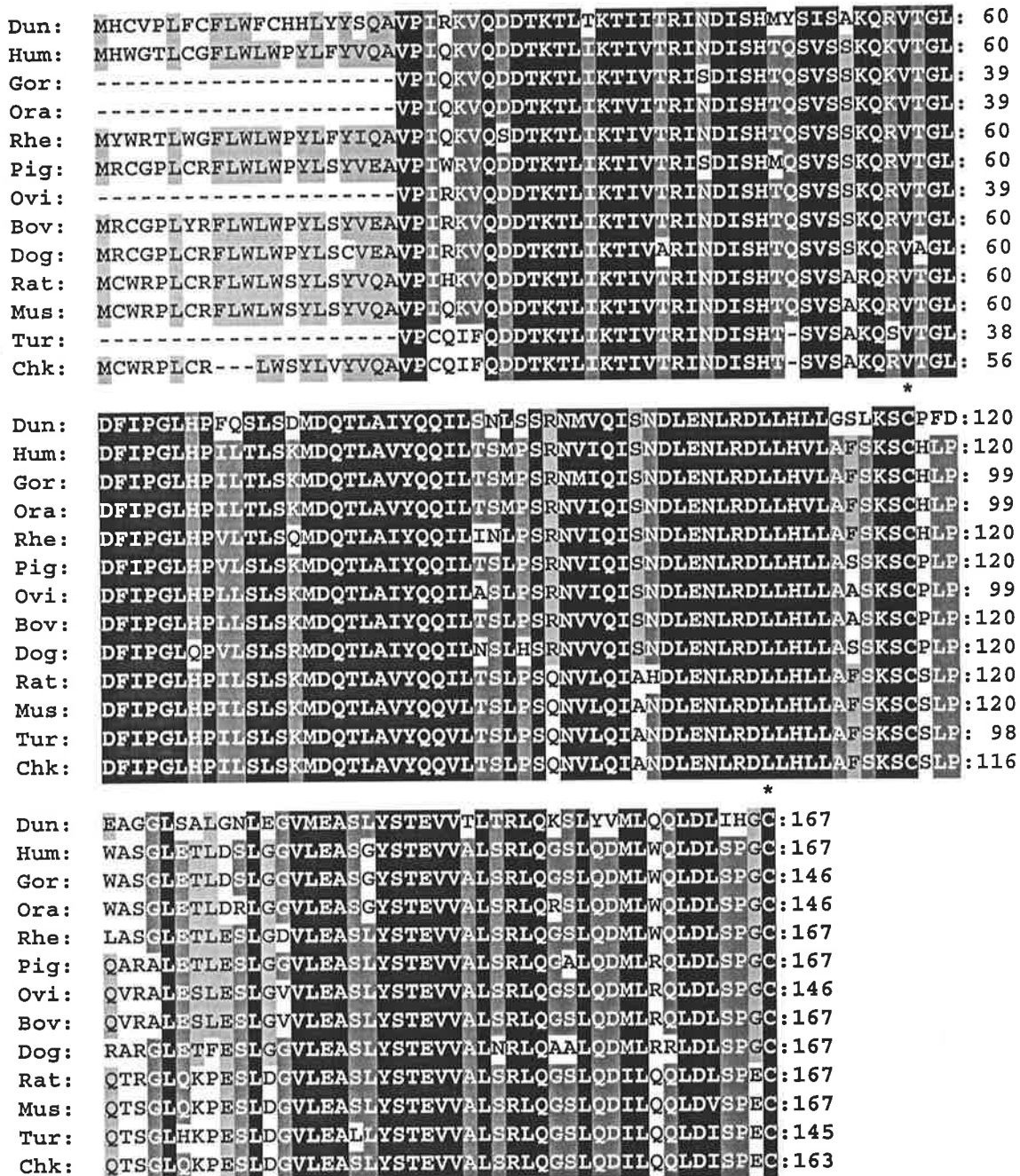


**Figure 6.2:** Amino acid sequence alignment of leptin from eleven species of mammals and two birds. The *S. crassicaudata* sequence (Dun), derived in this study, is shown at the top. Abbreviations used for the other species are: human (Hum), gorilla (Gor), orangutan (Ora), Rhesus monkey (Rhe), sheep (Ovi), cow (Bov), mouse (Mus), turkey (Tur) and chicken (Chk). Four levels of shading are used to indicate degrees of sequence similarity: black background with white letters = 100% similarity, grey background with white letters = 80% similar residues, grey background with black letters = 60% similar residues, white background with black letters = fewer than 60% similar residues. Conservative substitution groups are taken to be: (D, N), (E, Q), (S, T), (K, R), (F, Y, W), and (L, I, V, M). Putative signal peptides are boxed. The two conserved cysteine residues are topped by an asterisk.

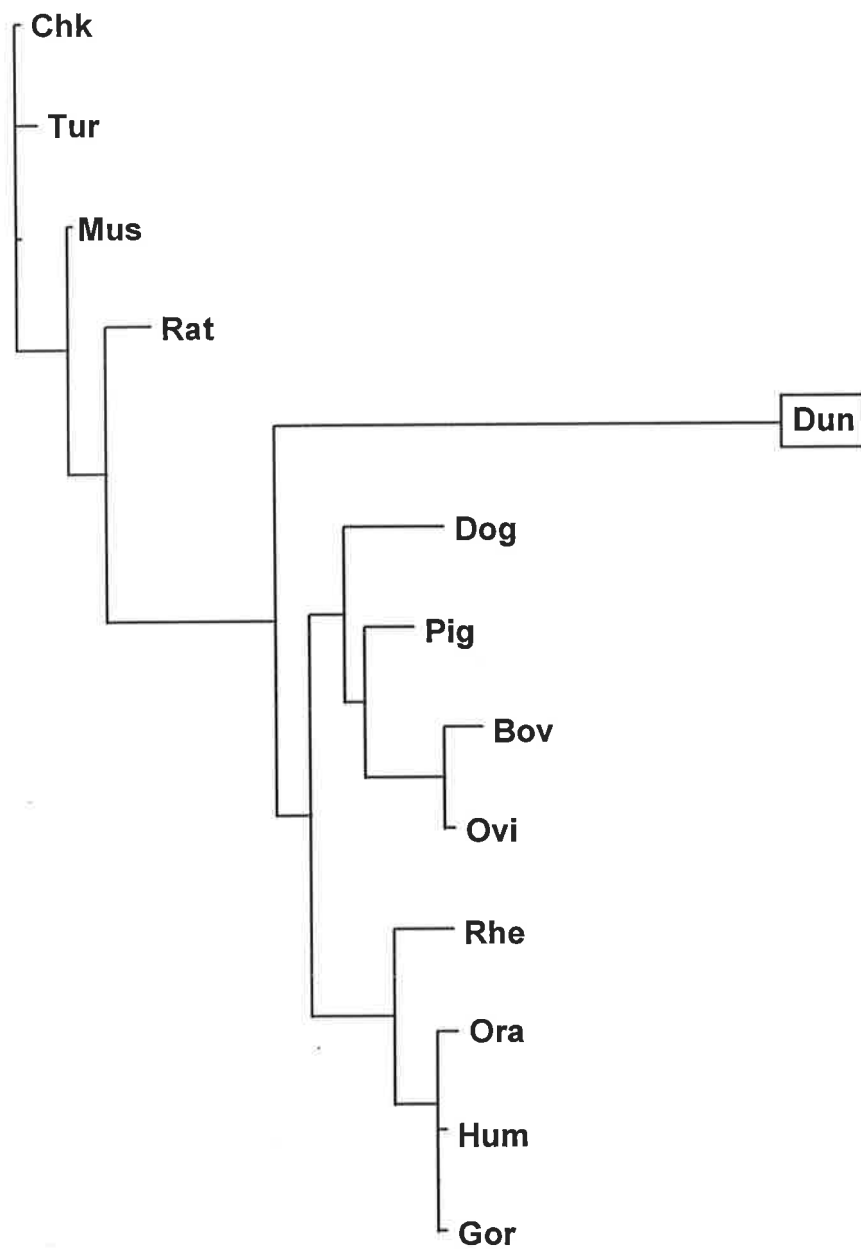


**Figure 6.3:** Maximum likelihood phylogenetic tree derived from aligned leptin cDNA sequences, using chicken and turkey as outgroups. Abbreviations as in figure 6.2. The branch lengths are drawn to scale.

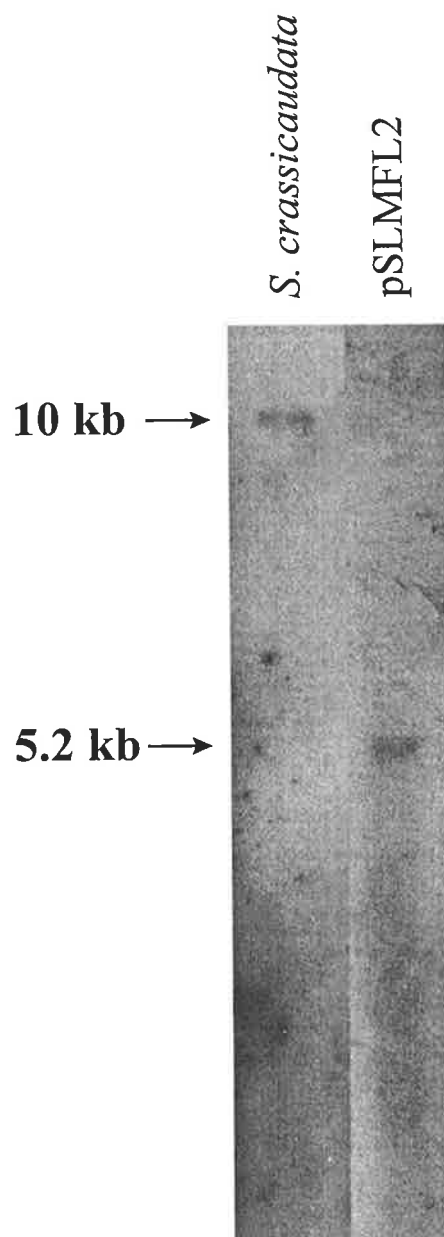


**Figure 6.4:** Southern blot of *S. crassicaudata* genomic DNA digested with EcoR1, and pSLMFL2 plasmid (containing the entire coding region of *S. crassicaudata* leptin), probed with the 1.8 kb cDNA for *S. crassicaudata* leptin. A single hybridisation band of ~ 10 kb was detected for the genomic DNA, and a single band of ~ 5.2 kb was detected for the undigested pSLMFL2 plasmid.

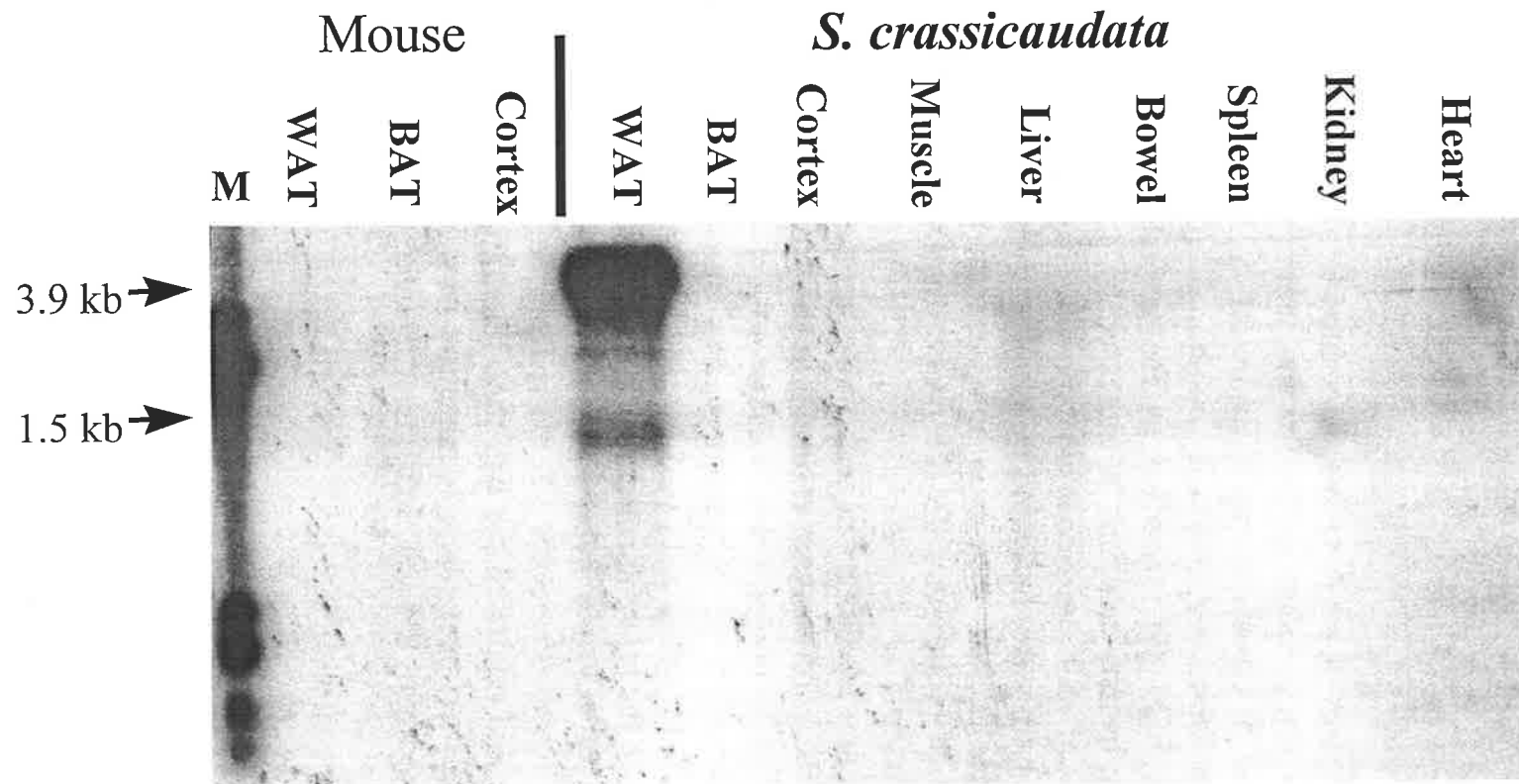
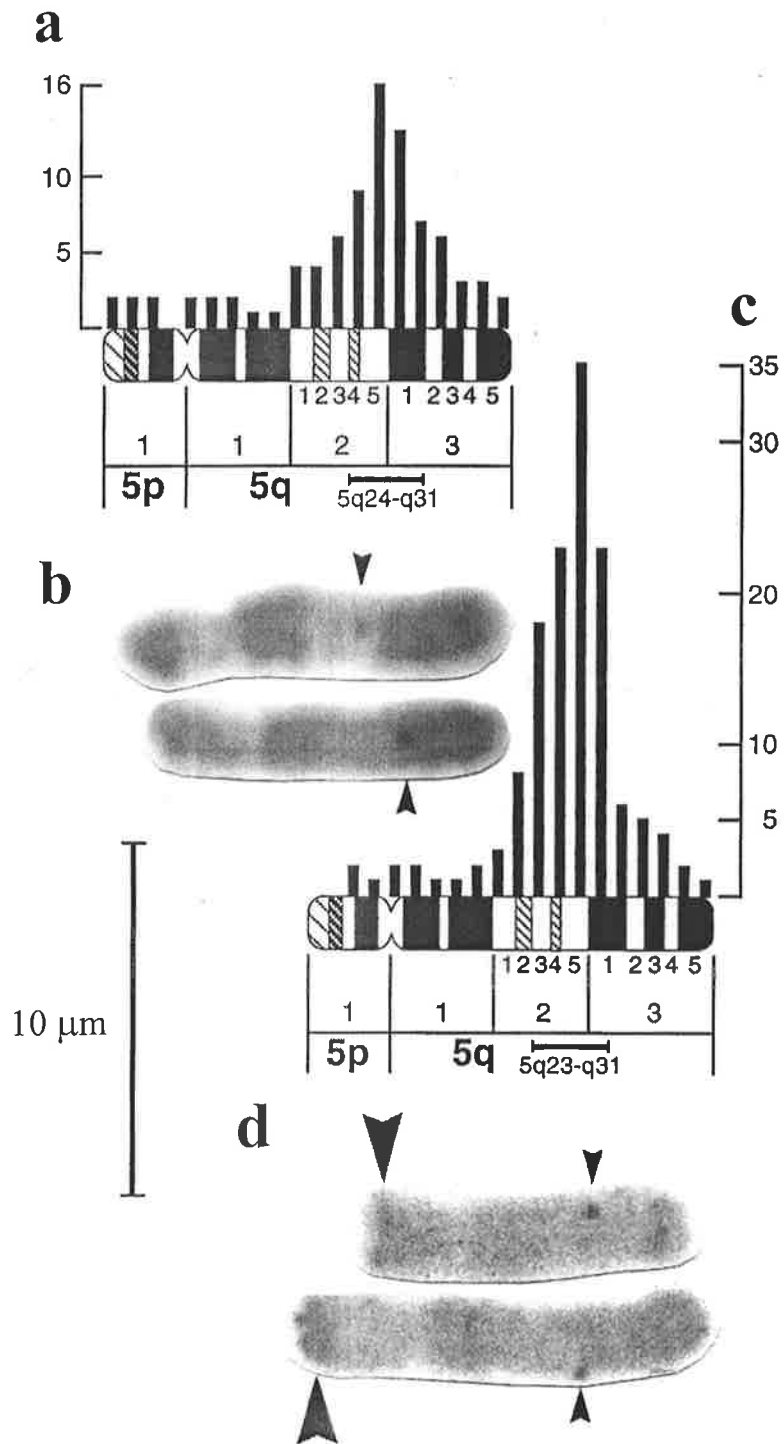


**Figure 6.5:** Northern blot of total RNA (20  $\mu$ g) from various tissues of *S. crassicaudata*, and mouse WAT and BAT, probed with labelled 1.8 kb cDNA for *S. crassicaudata* leptin. Hybridisation signal was only detectable in RNA from sub-cutaneous WAT adipose tissue deposits of *S. crassicaudata*, the transcript estimated to be  $\sim$  3.9 kb. Lane (M) contains molecular markers (Gibco-BRL 0.24-9.5 kb, Life Technologies).

**Figure 6.6:** (a) Scores of silver grains, generated by *in situ* hybridisation of a tritiated 1.8 kb genomic probe for the leptin gene, to R-banded chromosomes 5 of *S. crassicaudata*. The tallest column of silver grain signals is over the faint band arbitrarily-numbered 5q25. The arbitrarily-numbered region 5q24-q31, regarded as the probable location of the leptin gene, using tritium-labelling, is indicated by a line. (b) R-banded chromosomes 5 of *S. crassicaudata* showing signal silver grains attributed to the leptin gene, indicated by small arrowheads. (c) Scores of FITC signals under excitation by blue light, generated by fluorescent *in situ* hybridisation of biotinylated 1.8 kb probe for the leptin gene to R-banded chromosomes 5 of *S. crassicaudata*. This plot is similar to that obtained using tritium-labelling, but with more signals observed over the faint segment of the chromosome, resulting in the arbitrary region 5q23-q31 being regarded as the probable location of the leptin gene. (d) R-banded chromosomes 5 of *S. crassicaudata* showing FITC signals attributed to the leptin gene, indicated by small arrowheads. The larger arrowheads indicate the nucleolar-organising regions near the terminus of arm 5p, the site of multiple hybridisations of a biotinylated 5 kb 18S ribosomal probe. Notes: Prints for (d) made from colour-positive slides. The 10  $\mu$ M scale line relates to the half-tone illustrations. Both peaks of signal indicate that the most likely localisation of the leptin gene is the arbitrarily-numbered faint R-band, 5q25, near its interface with 5q31.





## 6.5 DISCUSSION

This study provides the first molecular information on leptin from a marsupial species. I have isolated and sequenced the cDNA for leptin from the marsupial *S. crassicaudata*, established that the leptin gene is located on chromosome 5, and shown that the mRNA for leptin is only expressed in white adipose tissue. Previous studies examining cross species hybridisation using mouse *ob* cDNA probes on a genomic Southern blot have resulted in detectable hybridisation signals in a large range of vertebrate species including chicken, cow, cat and rabbit (Zhang *et al* 1994). Further examinations of leptin and its molecular characteristics have demonstrated that the *ob* gene coding sequence shows high levels of evolutionary conservation, with human leptin being 84% similar to mouse at the protein level (Zhang *et al* 1994), and similarly high levels of conservation existing between a wide range of eutherian mammals. Examination of leptin from several species of birds has shown that they also share this high homology (Ashwell *et al* 1998, Taouis *et al* 1998).

This high level of evolutionary conservation of the *ob* gene suggests that the function of its protein product, leptin, is highly conserved. This appears to be the case, with both central and peripheral leptin administration having similar effects on the regulation of energy expenditure and food intake across a range of species including mouse, rat, dog and monkey (Pelleymounter *et al* 1995, Pelleymounter 1997, Weigle *et al* 1995). Mouse recombinant leptin administered peripherally decreases food intake and body weight in ground squirrels (*Spermophilus parryii*) (Boyer *et al* 1996), and human recombinant leptin administration increases energy expenditure,

and decreases food intake and body weight in *ob/ob* mice (Hwa *et al* 1997). Studies outlined in this thesis have demonstrated that peripheral administration of recombinant human leptin to *S. crassicaudata* induces a decrease in body weight, body fat and energy intake (see chapter 7), indicating that conservation of leptin function extends to dasyurid marsupials.

Despite strong conservation of structure across species, the *S. crassicaudata* cDNA probe did not hybridise to adipose tissue RNA from mouse. Failure of leptin probes to cross hybridise with the RNA from other species has been shown previously (Masuzaki *et al* 1995). Despite its failure to cross hybridise with mouse, the cDNA for *S. crassicaudata* leptin is structurally very similar to that of other species. As in all species examined to date, *S. crassicaudata* leptin cDNA has a very long 3'UTR (possibly effects mRNA stability), and contains two cysteine residues. The presence of these two cysteine residues is completely conserved across all species examined and they are accordingly thought to play an important structural role. In human leptin, these cysteine residues have been shown to be involved in an intrachain disulfide bridge that is essential for correct structure, stability and biological activity of the leptin protein (Rock *et al* 1996). Mutation of either of these cysteine residues results in biological inactivation of the protein (Zhang *et al* 1997).

The putative signal peptide of *S. crassicaudata* is similar in length to that of the human and mouse, however it is far less highly conserved than the mature protein. This suggests that the precise sequence of the signal peptide is not critical to protein secretion. Several residues in *S. crassicaudata* leptin differ from conserved residues

across other species. For example, residue 14 of the mature leptin protein is an isoleucine (Ile) in all other species studied, however in *S. crassicaudata* it is a threonine (Thr), Fig 6.2. By extending studies on leptin to other marsupial species it may be possible to identify regions of the protein that are conserved amongst marsupials but differ from the homologous regions in eutherians. Such regions would be good candidates for involvement in any 'marsupial-specific' functional characteristics of leptin.

An unexpected feature of the phylogenetic tree, Fig 6.3, and of a consensus maximum parsimony tree (data not shown) is that the two rodent species, rat and mouse, fall outside a monophyletic group comprising all other mammals including *S. crassicaudata*. A possible explanation for this anomaly is that the key assumption underlying the application of the algorithms used, namely that every site evolves independently (Felsenstein 1993), has been violated. Another explanation is that the leptin cDNAs compared are not from orthologous genes. Perhaps the most likely explanation, and one which finds support from the low bootstrap values obtained, is that the anomalous position of the rodents is due to chance. The high levels of sequence conservation in leptin cDNA, and the relatively short sequence length, result in there being few phylogenetically informative sites in the sequence alignments. Phylogenetic studies using the entire leptin gene, including introns, may help to resolve these issues. Exempting the rodents, the remainder of the phylogenetic relationships depicted in figure 6.3 are as expected, with the primates and non-primate eutherians falling into sister groups, to the exclusion of *S. crassicaudata*.

Although the leptin protein sequences are highly conserved, the phylogenetic tree, Fig 6.3 indicates that there has been more sequence divergence in the *S. crassicaudata* leptin lineage than in the other mammalian lineages. This observation was confirmed using the relative rates test (Wu & Li 1985), which estimates the rate of molecular evolution along each of two branches of a phylogenetic tree since a common ancestral sequence was last shared. The test requires no knowledge of actual divergence times. Using chicken leptin cDNA as an out-group, it was found that in every pair-wise sequence comparison involving *S. crassicaudata* and a eutherian, the *S. crassicaudata* leptin cDNA sequence had evolved at a significantly faster rate (all Z-scores less than - 4.19,  $P < 0.001$ ). None of the pair-wise eutherian sequence comparisons indicated significantly different rates of molecular evolution. This finding suggests that since the marsupial and eutherian leptin sequences diverged, they have been subjected to different selective pressures and that in the marsupial lineage these pressures have resulted in a relatively rapid rate of molecular evolution, potentially indicating one or more functional changes.

Every leptin gene so far reported has an intron located in the corresponding region within the coding sequence. In the mouse and human leptin genes, this intron occurs after amino acid residue 48, and is approximately 1.7 and 2.3 kb in length respectively (He *et al* 1995, Isse *et al* 1995). In preliminary studies to determine the presence of such an intron in *S. crassicaudata*, polymerase chain-reaction (PCR) on *S. crassicaudata* genomic DNA using primers that flank the putative intron insertion point has been carried out. The PCR product was identical in size (507 bp) to the corresponding region of cDNA, suggesting that this intron may be absent. If

confirmed, the absence of this intron would further indicate that marsupial leptin genes may have some unique features not shared by their eutherian counterparts.

Definitive karyograms have never been constructed for marsupials, in spite of the availability of good G-bands (Rofe 1979), although a rough un-numbered G-banded idiogram for *S. crassicaudata* has been published (Graves *et al* 1990). The present numbering of the R-bands of chromosome 5 must be taken to only apply to this work. The leptin gene clearly localises to the long arm of chromosome 5 in *S. crassicaudata*, to the arbitrarily numbered sub-bands 5q23-q31. Peaks of signal indicate that the most likely point localisation of the leptin gene is the arbitrarily numbered faint R-band, 5q25, near its interface with 5q31. The increased signals, above background, on chromosome 6 might be due to some cross hybridisation of the leptin gene to similar DNA sequences. With tritium labelling, there was similar signal peaks on each arm of the metacentric 6, which may indicate some miss-identification of the arms. If the peaks over background are added for chromosome 6, the result might be judged as generated from a leptin pseudogene (Webb *et al* 1990), but this is not supported by the single band on Southern blots. In addition to physically assigning the leptin gene, the single NOR of *S. crassicaudata* was found to be probably sub-distal on the short arm of chromosome 5.

Footnote: The nucleotide sequence reported in this chapter has been submitted to the GenBank Data Bank, accession number AF159713.

## CHAPTER 7

**THE EFFECT OF DIET ON THE RESPONSE TO LEPTIN IN  
*S. CRASSICAUDATA*****7.1 SUMMARY**

Plasma leptin levels have been demonstrated to increase with increasing adiposity and in diet-induced obese rodents, increased leptin levels are associated with reduced sensitivity to leptin administration. Studies presented in chapter 5 have demonstrated that diet-induced obesity can occur in the marsupial *S. crassicaudata* in response to feeding the high calorie, higher fat diet of mealworms, as opposed to standard laboratory diet. The effect of leptin on food intake and fat mass in marsupials has not previously been determined. The aim of this study was to determine in *S. crassicaudata* i) the effect of chronic leptin administration on food intake, body fat stores and metabolism, and ii) whether leptin can prevent a diet-induced increase in adiposity. *S. crassicaudata* were randomly allocated to receive either standard laboratory diet or a choice between laboratory diet and mealworms. For 13 days, half the animals in each dietary group received intraperitoneal (IP) human leptin (2.5 mg/kg twice daily), while the other half received PBS.

In animals receiving laboratory diet alone, leptin induced a decrease in body weight ( $P < 0.0001$ ), tail width ( $P < 0.0001$ ) and energy intake ( $P < 0.01$ ). In animals receiving

both laboratory diet and mealworms, leptin had no effect on body weight or tail width, although the proportion of laboratory diet eaten was reduced ( $P = 0.0001$ ), and there was a non-significant fall in overall energy intake ( $P = 0.07$ ). It can be concluded that, although leptin induces weight loss in *S. crassicaudata*, an increase in dietary calories and fat is associated with resistance to the actions of leptin.

## 7.2 INTRODUCTION

The *ob* protein leptin circulates in proportion to total body fat mass (Maffei *et al* 1995), and plasma leptin levels rise with increasing adiposity in both rodents and humans (Considine *et al* 1996, Frederich *et al* 1995a, Lonnqvist *et al* 1995). The reason for the failure of elevated leptin levels, associated with weight gain, to restrain large increases in weight is not known; this has led to the suggestion that leptin resistance occurs (see section 1.5.4).

The mechanisms for leptin resistance are uncertain. It has been suggested that leptin may have a limited ability to restrain intake of highly palatable, high fat diets that are normally associated with an increase in adiposity (Widdowson *et al* 1997). Previous studies in mice suggest that defects downstream of the leptin receptor in the hypothalamus may account for leptin resistance (Halaas *et al* 1997). The observation that in obese humans elevated plasma leptin is not associated with an increase in cerebrospinal fluid levels of leptin, has led to the hypothesis that the leptin transport system may be defective or saturated in obese humans (Caro *et al* 1996, Schwartz *et*

*al* 1996a). The finding that C57BL/6 and AKR diet-induced obese mice have preserved sensitivity to centrally administered leptin, despite resistance to peripherally administered leptin, is consistent with this concept (VanHeek *et al* 1997).

The leptin gene from the marsupial *S. crassicaudata* has now been cloned (see chapter 6), however it is not known whether leptin regulates food intake and body fat mass in marsupials as in other mammals. Studies presented in chapter 11 have demonstrated that *S. crassicaudata* prefer a diet of mealworms to their standard laboratory diet, and it has also been demonstrated that that this preferred diet induces an increase in adiposity, not unlike 'cafeteria' feeding in rats (see chapter 5). Whether the increased adiposity of *S. crassicaudata* in response to the preferred diet of mealworms is associated with leptin resistance is unknown.

Therefore, the aim of this study was to determine in *S. crassicaudata* the effect of peripheral leptin administration on body weight, tail width (fat mass) and food intake, when animals are fed laboratory diet alone (low calorie), compared to when they are given a choice between laboratory diet and the preferred higher calorie, higher fat diet of mealworms.



## 7.3 MATERIALS AND METHODS

### 7.3.1 *Experimental animals, diets and drugs*

Adult male *S. crassicaudata* housed under a long-day light regime (LD; 16-h light, 8-h dark; lights off 16:00h). Standard laboratory diet was provided *ad libitum* to all animals unless specified. Mealworms were provided *ad libitum* where indicated in the experimental protocols. Data for food intake are expressed as kcal/g of body weight or total kcal.

Purified r-met human leptin was prepared as stated in section 3.5.1. PBS vehicle or leptin (2.5 mg/kg) were administered intraperitoneally (IP).

### 7.3.2 *Metabolic parameters*

Oxygen consumption ( $\text{VO}_2$ ) and respiratory quotient (RQ) were determined at 24 °C using established methods described in section 3.3.4. 'Baseline'  $\text{VO}_2$  for each animal was defined as the most stable value reached at least 15 minutes after placement of the animal into the chamber, with the animal sitting motionless.

### 7.3.3 *Experimental protocols*

Study 1: To determine i) the chronic effect of leptin on body weight, tail width, body temperature, food intake, oxygen consumption and RQ, and ii) whether these responses are modified by diet.

*S. crassicaudata* were divided into 4 groups of similar initial body weight ( $n = 9-10$  per group). Body weight, tail width (see 3.3.1), body temperature (see 3.3.3) and

food intake (see 3.3.2) were measured daily for 5 days. In each animal, PBS was injected (IP) at 16:00h on day 5, at 08:30h and 16:00h on *day 6*, and at 08:30h on *day 7*. On *day 7* at 16:00h, half of the animals continued to receive PBS and half were injected (IP) with purified r-met human leptin in a dose of 2.5 mg/kg. Animals received twice daily (08:30h and 16:00h) (IP) injections of PBS or leptin for a further 12 days. In each of the PBS and leptin treated groups, half the animals were fed with laboratory diet only, and half with a combination of laboratory diet and mealworms. 'Baseline'  $\text{VO}_2$  and RQ were determined for each animal on *day 5*, *day 13* (*ie.* 7 days after randomisation to leptin or PBS) and *day 19* (*ie.* after 12 days of leptin treatment). Body weight, tail width, body temperature and food intake were measured daily at 15:30h. To determine if there were any acute effects of leptin on food consumption, food intake was recorded in each animal at 0.5, 1, 2, 4 and 24 hours following the 16:00h injection on *days 7* and *15*.

Study 2: *Effect of leptin on blood glucose concentration and whether this response is modified by diet.*

The protocol was similar to that used in study 2 above. Baseline blood glucose was determined on *day 5* at 08:30h in 24 animals, using the method described in sections 3.4 and 3.4.1. Animals were then divided into 3 groups ( $n = 8$ ) matched for initial body weight and blood glucose concentration. Injections of PBS were given as in experiment 2. On *day 7* at 16:00h, one group of animals received (IP) leptin at 2.5mg/kg and was then re-fed with laboratory diet and mealworms. The other 2 groups received PBS, one of these then received laboratory diet and the other both laboratory diet and mealworms. Leptin or PBS injections were continued twice daily

for a further 12 days. The blood glucose concentration was measured at 08:30h on *days 8 and 18*.

#### 7.3.4 *Statistical Analyses*

Analyses were performed as stated in section 3.9.

## 7.4 RESULTS

### 7.4.1 Study 1: Effect of leptin on body weight and tail width in relation to diet

#### 7.4.1.1 *Cumulative food intake*

ANIMALS FED LABORATORY DIET ONLY. Cumulative food intake did not differ significantly over the baseline period (*days 1-7*) between the ‘PBS’ ( $85.3 \pm 8.69$  kcal) or ‘leptin’ allocated groups ( $85.36 \pm 7.62$  kcal). Administration of leptin resulted in a 17.9% decrease in food intake, and between *days 7-19* cumulative food intake was  $176.0 \pm 16.9$  kcal and  $146.3 \pm 12.7$  kcal in the PBS and leptin treated groups respectively, (drug  $F_{1,233} = 2.25$ ,  $P = 0.15$ , drug x day  $F_{12,233} = 2.3$ ,  $P = 0.009$ ), Fig 7.1A.

ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS. Cumulative food intake did not differ significantly over the baseline period (*days 1-7*) between the ‘PBS’ ( $94.91 \pm 5.35$  kcal) or ‘leptin’ allocated groups ( $80.65 \pm 5.25$  kcal), (drug  $F_{1,132} = 2.14$ ,  $P = 0.16$ ). There was, however, a significant drug x day interaction, (drug x

day  $F_{6,132} = 4.48$ ,  $P = 0.0005$ ). Leptin had no significant effect on total food intake, and between *days 7 and 19* cumulative food intake was  $217.5 \pm 16.5$  kcal and  $190.0 \pm 14.9$  kcal in the PBS and leptin treated groups respectively, (drug  $F_{1,246} = 1.08$ ,  $P = 0.31$ , drug x day  $F_{12,246} = 1.68$ ,  $P = 0.07$ ), Fig 7.1B.

#### FOOD CHOICE IN ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS.

In animals fed both laboratory diet and mealworms there was a significant reduction in the intake of laboratory diet in leptin treated animals compared to controls. Cumulative intake of laboratory diet from *days 7-19* was  $135.77 \pm 26.05$  kcal and  $75.87 \pm 23.18$  kcal in the PBS and leptin treated groups respectively, (drug  $F_{1,246} = 3.66$ ,  $P = 0.07$ , drug x day  $F_{12,246} = 2.75$ ,  $P = 0.002$ ), Fig 7.2A. There was a non-significant increase in mealworm intake between the two groups ( $P = 0.09$ ). Cumulative mealworm intake from *days 7-19* was  $81.71 \pm 24.65$  kcal and  $111.25 \pm 30.74$  kcal in the PBS and leptin treated groups respectively, Fig 7.2B.

##### 7.4.1.2 *Body weight*

ANIMALS FED LABORATORY DIET ONLY. Body weight did not change over the baseline period (between *days 1 and 7*) in either the 'PBS' ( $15.13 \pm 0.36$  g vs  $15.51 \pm 0.44$  g) or 'leptin' allocated groups ( $14.87 \pm 0.38$  g vs  $14.62 \pm 0.44$  g), and was not significantly different between the two groups. Administration of leptin was associated with a fall in body weight of  $8.34 \pm 0.42\%$ , and by *day 19*, body weight was  $15.25 \pm 0.26$  g and  $13.4 \pm 0.41$  g in the PBS and leptin treated groups respectively, (drug  $F_{1,341} = 7.41$ ,  $P = 0.02$ , drug x day  $F_{18,341} = 5.44$ ,  $P < 0.0001$ ), Fig 7.3A.

**ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS.** Body weight did not change over the baseline period (between *days 1* and *7*) in either the ‘PBS’ ( $14.63 \pm 0.44$  g vs  $14.62 \pm 0.54$  g) or ‘leptin’ allocated groups ( $14.51 \pm 0.43$  g vs  $15.04 \pm 0.50$  g), and body weight was not significantly different between the two groups. After commencement of the diets, both PBS and leptin treated animals showed a significant increase in body weight from baseline ( $P < 0.05$ ), and on *day 19* body weight was  $15.84 \pm 0.51$  g and  $15.41 \pm 0.53$  g in the PBS and leptin treated animals respectively, Fig 7.3B.

#### 7.4.1.3 Tail width

**ANIMALS FED LABORATORY DIET ONLY.** Tail width did not change over the baseline period (between *days 1* and *7*) in either the ‘PBS’ ( $4.20 \pm 0.14$  mm vs  $4.19 \pm 0.12$  mm) or ‘leptin’ allocated groups ( $4.33 \pm 0.38$  mm vs  $4.32 \pm 0.22$  mm), and there was no significant difference between these groups. Leptin administration was associated with a decrease in tail width of  $17.1 \pm 0.1\%$  and by *day 19*, tail width was  $4.00 \pm 0.14$  mm and  $3.58 \pm 0.20$  mm in the PBS and leptin treated groups respectively, (drug  $F_{1,341} = 0.25$ ,  $P = 0.62$ , drug x day  $F_{18,341} = 6.80$ ,  $P < 0.0001$ ), Fig 7.3C.

**ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS.** Tail width did not change over the baseline period (between *days 1* and *7*) in either the ‘PBS’ ( $3.98 \pm 0.14$  mm vs  $3.95 \pm 0.15$  mm) or ‘leptin’ allocated groups ( $3.96 \pm 0.19$  mm vs  $3.97 \pm 0.13$  mm), and there was no significant difference between the groups. Leptin administration had no significant effect on tail width and on *day 19* tail width was

4.13  $\pm$  0.18 mm and 3.96  $\pm$  0.16 mm in the PBS and leptin treated groups respectively, Fig 7.3D.

#### 7.4.1.4 *Body temperature*

**ANIMALS FED LABORATORY DIET ONLY.** Body temperature did not change over the baseline period (between *days 1* and *7*) in either the ‘PBS’ (34.52  $\pm$  0.29 °C vs 34.67  $\pm$  0.21 °C) or ‘leptin’ allocated groups (35.10  $\pm$  0.24 °C vs 34.91  $\pm$  0.24 °C), and there was no significant difference between the groups. On *day 19* body temperature was 32.41  $\pm$  0.68 °C and 32.78  $\pm$  0.72 °C in the PBS and leptin treated groups respectively, and did not differ significantly from baseline.

**ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS.** Body temperature did not change over the baseline period (between *days 1* and *7*) in either the ‘PBS’ (34.71  $\pm$  0.34 °C vs 34.67  $\pm$  0.23 °C) or ‘leptin’ allocated groups (34.28  $\pm$  0.27 °C vs 34.29  $\pm$  0.18 °C), and body temperature did not differ significantly between these groups. On *day 19* body temperature was 32.84  $\pm$  0.48 °C and 33.57  $\pm$  0.52 °C in the PBS and leptin treated groups respectively, and did not differ significantly from baseline.

#### 7.4.1.5 *Metabolic parameters*

**ANIMALS FED LABORATORY DIET ONLY.** At baseline (*day 5*), VO<sub>2</sub> and RQ did not differ between the ‘PBS’ and ‘leptin’ allocated groups. Administration of leptin had no significant effect on VO<sub>2</sub> compared to PBS treated controls, on either *days 13* or *19*. On *day 13* RQ’s were not significantly different from baseline,

however on *day 19*, leptin treated animals had a significantly higher RQ than the PBS treated controls,  $P = 0.05$ , Table 7.1.

**ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS.** At baseline (*day 5*),  $VO_2$  and RQ did not differ between the 'PBS' and 'leptin' allocated groups. On *day 19*,  $VO_2$  of the leptin treated animals was less than that of the PBS treated controls,  $P = 0.04$ . RQ was similar in the PBS and leptin treated groups on *days 13* and *19*, Table 7.1.

#### 7.4.1.6 *Effect of leptin on short-term food intake and food choice*

**ANIMALS FED LABORATORY DIET ONLY.** Leptin administration had no significant effect on subsequent 24-hour food intake (laboratory diet) at any of the time points studied, on either *day 7* following the first leptin injection, or on *day 15*, after 9 days of leptin treatment, Table 7.2.

**ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS.** Leptin administration had no significant effect on subsequent 24-hour food intake (both laboratory diet and mealworms) at any of the time points studied, on either *day 7* following the first leptin injection, or on *day 15*, after 9 days of leptin treatment, Table 7.2.

**FOOD CHOICE IN ANIMALS FED BOTH LABORATORY DIET AND MEALWORMS.** On *day 7* following the first leptin injection, leptin reduced intake of laboratory diet over the next 24 hours when compared to the PBS control group,

(drug  $F_{1,94} = 3.5$ ,  $P = 0.08$ , drug x time  $F_{4,94} = 5.10$ ,  $P = 0.001$ ), Fig 7.4A. On *day 15*, after 8 days of leptin treatment, leptin had no significant effect on the intake of laboratory diet at any of the time points studied, (drug  $F_{1,94} = 2.75$ ,  $P = 0.12$ , drug x time  $F_{4,94} = 1.92$ ,  $P = 0.12$ ), Fig 7.4B. When compared to the PBS treated animals, leptin had no significant effect on the subsequent 24-hour intake of mealworms on either *day 7* following the first leptin injection, (drug  $F_{1,94} = 2.07$ ,  $P = 0.17$ , drug x time  $F_{4,94} = 2.24$ ,  $P = 0.07$ ), Fig 7.4C, or on *day 15* after 8 days of leptin treatment, (drug  $F_{1,94} = 1.02$ ,  $P = 0.33$ , drug x time  $F_{4,94} = 0.90$ ,  $P = 0.47$ ), Fig 7.4D.

#### 7.4.2      Study 2: Effect of leptin on blood glucose concentration in relation to diet

Administration of leptin had no effect on blood glucose concentrations. Blood glucose levels did not differ significantly at any of the time points studied between the three groups, Table 7.3.



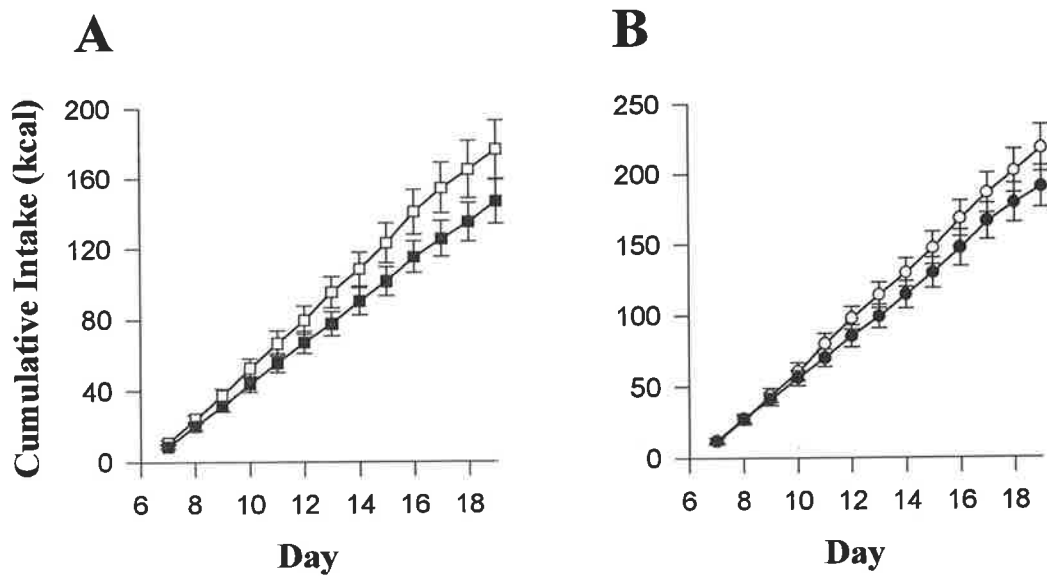


**Figure 7.1:** Cumulative energy intake (kcal) of long-day housed (16-h light, 8-h dark) *S. crassicaudata* (from days 7 to 19) given IP injections of PBS or leptin (2.5mg/kg) twice daily and re-fed with either laboratory diet alone (A) or with a choice between laboratory diet and mealworms (B). Leptin administration induced a decrease in energy intake in animals given laboratory diet only ( $P = 0.009$ ), and had no effect on energy intake in those animals given both laboratory diet and mealworms. Values are mean  $\pm$  SE. □, PBS + laboratory diet; ■, leptin + laboratory diet; ○, PBS + laboratory diet and mealworms; ●, leptin + laboratory diet and mealworms.

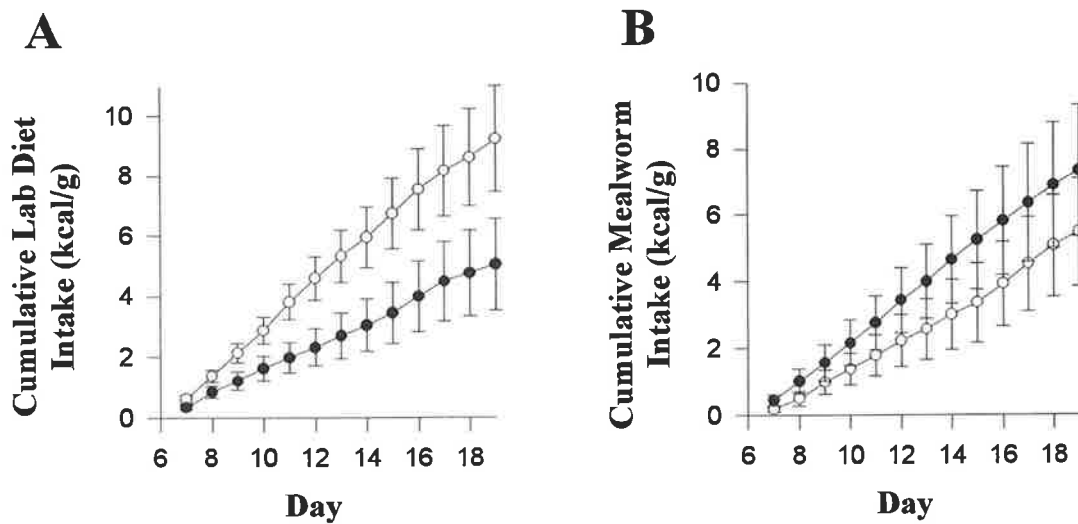


**Figure 7.2:** Cumulative energy intake (kcal/g) of laboratory diet (A) and mealworms (B) of long-day housed (16-h light, 8-h dark) *S. crassicaudata* (from *days 7 to 19*) given IP injections of PBS or leptin (2.5mg/kg) twice daily and re-fed with a choice between laboratory diet and mealworms. Laboratory diet intake decreased in response to leptin administration ( $P = 0.02$ ), and there was a non-significant increase in mealworm intake ( $P = 0.09$ ). Values are mean  $\pm$  SE.  $\circ$ , PBS;  $\bullet$ , leptin.

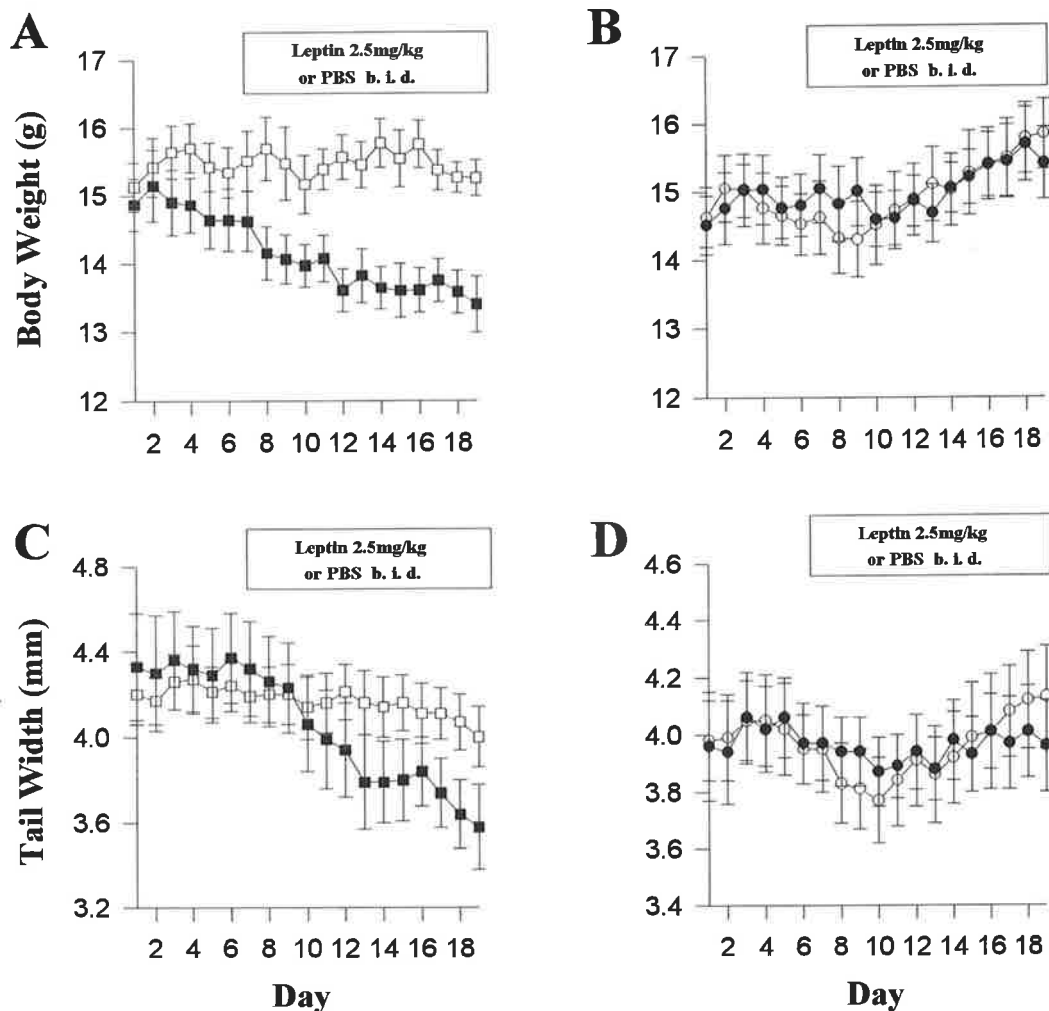


**Figure 7.3:** Body weights (g; A and B) and tail widths (mm; C and D) of *S. crassicaudata* given IP injections of PBS or leptin (2.5mg/kg) twice daily and re-fed from *day 7* with either laboratory diet alone (A and C) or with a choice between laboratory diet and mealworms (B and D). Baseline body weights and tail widths were measured from *days 1* to *6*, and the first leptin injection was given on *day 7*. Placebo injections of PBS were given twice daily for 2 days before the first leptin injection. Leptin administration induced a decrease in both body weight and tail width when animals were fed laboratory diet alone ( $P < 0.0001$ ). In animals given both laboratory diet and mealworms leptin administration had no effect on body weight or tail width when compared to the PBS treated controls. Values are mean  $\pm$  SE.  $\square$ , PBS + laboratory diet;  $\blacksquare$ , leptin + laboratory diet;  $\circ$ , PBS + laboratory diet and mealworms;  $\bullet$ , leptin + laboratory diet and mealworms. b.i.d., Twice daily.

| Diet                                   | Oxygen Consumption, ml/g/h |             |               | Respiratory Quotient |             |               |
|----------------------------------------|----------------------------|-------------|---------------|----------------------|-------------|---------------|
|                                        | Day 5                      | Day 13      | Day 19        | Day 5                | Day 13      | Day 19        |
| PBS + laboratory diet                  | 4.19 ± 0.25                | 4.26 ± 0.38 | 5.02 ± 0.25   | 0.75 ± 0.02          | 0.75 ± 0.03 | 0.73 ± 0.02   |
| Leptin + laboratory diet               | 4.01 ± 0.30                | 4.71 ± 0.46 | 5.51 ± 0.52   | 0.74 ± 0.02          | 0.74 ± 0.02 | 0.81 ± 0.04 * |
| PBS + laboratory diet and mealworms    | 4.28 ± 0.22                | 4.53 ± 0.19 | 5.39 ± 0.21   | 0.74 ± 0.02          | 0.74 ± 0.02 | 0.70 ± 0.01   |
| Leptin + laboratory diet and mealworms | 4.22 ± 0.30                | 4.75 ± 0.47 | 4.47 ± 0.35 * | 0.79 ± 0.02          | 0.74 ± 0.03 | 0.75 ± 0.02   |

**Table 7.1:** Oxygen consumption and respiratory quotient in long-day housed (16-h light, 8-h dark) *S. crassicaudata* given intraperitoneal injections of PBS vehicle or leptin (2.5mg/kg) twice daily and re-fed from *day 7* with either laboratory diet alone or with a choice between laboratory diet and mealworms. Baseline metabolic parameters were measured on *day 5*, and the first leptin injection was given on *day 7*. Placebo injections of PBS were given twice daily for 2 days before the first leptin injection. Values are means ± SE. \* P < 0.05 vs. baseline.

| Diet                          | Time Post-injection, h | Cumulative Food Intake, kcal |                        |                      |                         |
|-------------------------------|------------------------|------------------------------|------------------------|----------------------|-------------------------|
|                               |                        | PBS<br><i>Day 7</i>          | Leptin<br><i>Day 7</i> | PBS<br><i>Day 15</i> | Leptin<br><i>Day 15</i> |
| Laboratory diet               | 0.5                    | 0.93 ± 0.14                  | 0.99 ± 0.18            | 1.91 ± 0.50          | 1.97 ± 0.39             |
|                               | 1                      | 2.24 ± 0.42                  | 1.83 ± 0.32            | 2.60 ± 0.53          | 2.79 ± 0.46             |
|                               | 2                      | 3.29 ± 0.50                  | 2.61 ± 0.41            | 4.31 ± 0.73          | 4.40 ± 0.84             |
|                               | 4                      | 5.31 ± 0.72                  | 4.58 ± 0.74            | 6.07 ± 0.85          | 5.94 ± 1.10             |
|                               | 24                     | 10.86 ± 1.47                 | 9.03 ± 1.14            | 14.75 ± 1.73         | 11.53 ± 2.13            |
| Laboratory diet and mealworms | 0.5                    | 0.95 ± 0.28                  | 1.07 ± 0.24            | 0.85 ± 0.35          | 1.70 ± 0.64             |
|                               | 1                      | 2.00 ± 0.44                  | 2.10 ± 0.49            | 1.58 ± 0.43          | 2.35 ± 0.59             |
|                               | 2                      | 3.59 ± 0.67                  | 3.72 ± 0.93            | 4.20 ± 0.77          | 3.82 ± 0.72             |
|                               | 4                      | 6.45 ± 1.27                  | 5.44 ± 1.09            | 7.59 ± 1.02          | 6.29 ± 0.99             |
|                               | 24                     | 13.0 ± 1.91                  | 12.32 ± 1.84           | 16.27 ± 1.84         | 15.56 ± 1.71            |

**Table 7.2:** Cumulative short-term food intake (kcal) in long-day housed (16-h light, 8-h dark) *S. crassicaudata* given an intraperitoneal injection of PBS vehicle or leptin (2.5mg/kg) on *day 7* (following the first leptin injection) and on *day 15* (after 8 days of leptin treatment). Injections were given at lights out, and animals were re-fed with laboratory diet only or a choice between laboratory diet and mealworms. Food intake was measured 0.5, 1, 2, 4, 6, and 24 h after injections. Values are means ± SE.

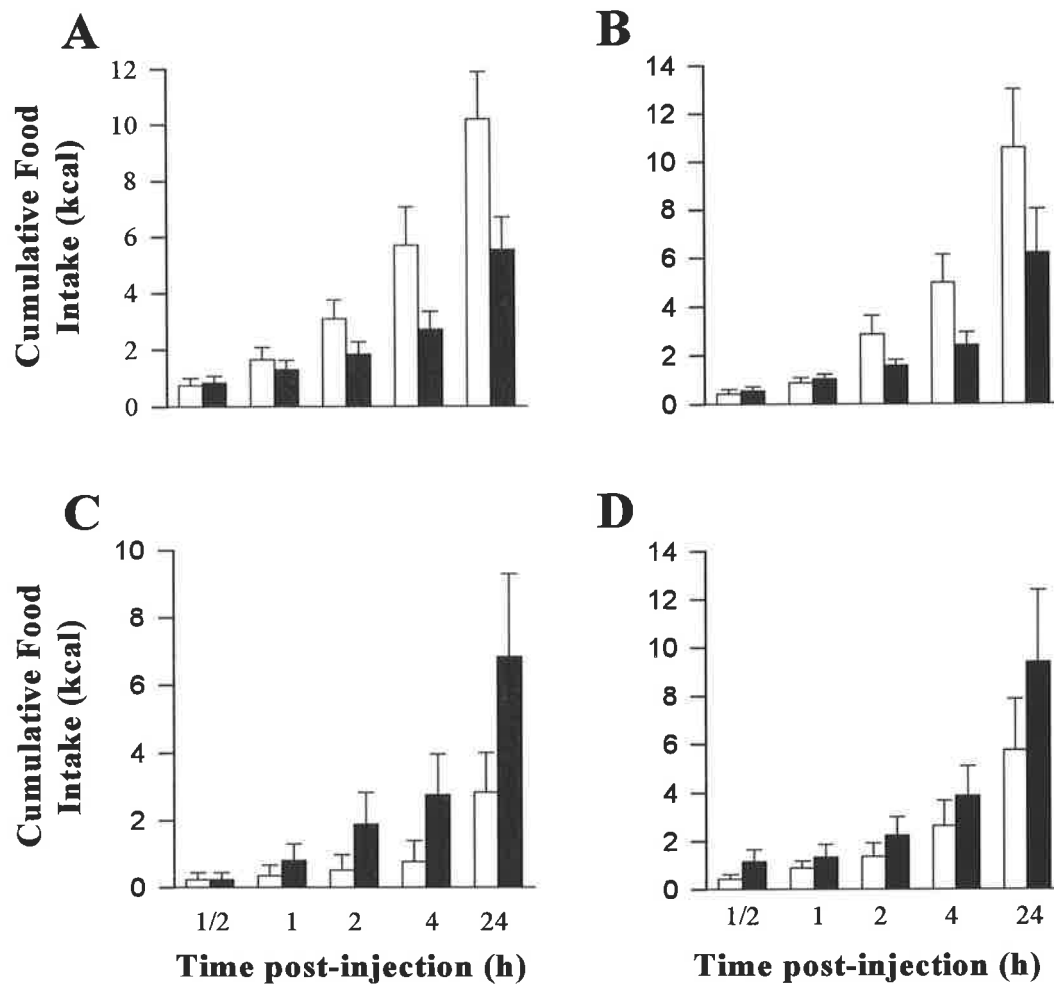


**Figure 7.4:** Food choice in long-day housed (16-h light, 8-h dark) *S. crassicaudata* given IP PBS or leptin (2.5mg/kg) and re-fed with a choice between laboratory diet and mealworms. Cumulative intake of laboratory diet (kcal) on *day 7*, after the first leptin injection (A), and on *day 15*, after 8 days of leptin treatment (B), and cumulative intake of mealworms (kcal) on *day 7*, after the first leptin injection (C), and on *day 15*, after 8 days of leptin treatment (D), are shown. Injections were given at lights out, and food intake was measured 0.5, 1, 2, 4, 6, and 24-h after injections. Leptin decreased laboratory diet intake on *day 7*, after the first leptin injection ( $P = 0.001$ ). Values are mean  $\pm$  SE. Open bars, PBS; filled bars, leptin.

| Diet                                   | Blood Glucose Concentration (mmol/l) |             |             |
|----------------------------------------|--------------------------------------|-------------|-------------|
|                                        | Day 4                                | Day 12      | Day 19      |
| PBS + laboratory diet                  | 3.80 ± 0.18                          | 4.25 ± 0.3  | 3.46 ± 0.30 |
| PBS + laboratory diet and mealworms    | 4.17 ± 0.24                          | 4.09 ± 0.31 | 3.46 ± 0.28 |
| Leptin + laboratory diet and mealworms | 3.89 ± 0.17                          | 4.30 ± 0.33 | 3.44 ± 0.18 |

**Table 7.3:** Blood glucose concentration of long-day housed (16-h light, 8-h dark) *S. crassicaudata* given intraperitoneal injections of PBS vehicle or leptin (2.5mg/kg) twice daily and re-fed from *day 7* with either laboratory diet alone or with a choice between laboratory diet and mealworms. Baseline blood glucose concentration was measured on *day 4*, and the first leptin injection was given on *day 7*. Placebo injections of PBS were given twice daily for 2 days before the first leptin injection. Values are mean ± SE.

## 7.5 DISCUSSION

Studies presented in chapter 5 have demonstrated that in the marsupial *S. crassicaudata*, body weight and fat mass (tail width) increase over 3 weeks, when fed a highly palatable, preferred diet of mealworms, which are calorie dense and have a greater fat and protein content than standard laboratory diet. This study demonstrates that while leptin administration induces a decrease in body weight and fat mass in animals fed standard laboratory diet, leptin fails to prevent an increase in body weight or tail fat stores when animals are fed both laboratory diet and mealworms. These data suggest that an increased intake of dietary fat, protein, and calories, results in increased adiposity, associated with resistance to the effect of exogenously administered leptin.

The effects of leptin on body weight and fat mass in both lean and genetically obese rodent models have been studied extensively. In the *ob/ob* leptin-deficient mouse, leptin administration caused a reduction in food intake of up to 40% within 4 days, and this was associated with a dramatic fall in body weight of approximately 40% within 33 days primarily due to a decrease in fat mass (Halaas *et al* 1995). Leptin has also been shown to decrease body weight and fat mass in the monkey and dog (Pelleymounter 1997). In wild type mice, leptin administration decreases fat mass from 12.2% to 0.67% of total body mass (Halaas *et al* 1995). In *S. crassicaudata* fed standard laboratory diet, leptin administration induced a decrease in body weight and food intake. The observation that tail width, a reflection of fat stores (see chapter 5),



decreased suggests that fat mass was reduced. This is the first demonstration that leptin can induce a decrease in food intake, body weight and fat mass in marsupials.

Leptin has been shown to have both short term (Campfield *et al* 1995) and relatively sustained (VanHeek *et al* 1997) effects on energy intake. In *S. crassicaudata*, as well as inducing an overall decrease in cumulative energy intake, leptin administration had acute effects on food consumption. When fed laboratory diet alone, leptin induced a 17% and 22% reduction in 24-hour food intake on *days 7* and *15* respectively. Although this did not reach statistical significance, the findings compare with a 10% decrease in 24-hour food intake in lean mice following leptin administration (Campfield *et al* 1995).

We cannot necessarily conclude that either the increase in calories, or the effect of any specific macronutrient in the mealworm diet was responsible for the observed effects, since the diets were markedly different not only in fat content but also in protein content. In addition, as mealworms are highly palatable to *S. crassicaudata* (see chapter 11), there remains the possibility that food intake was prejudiced by dietary preference. Our data are consistent with previous findings in rodents (Frederich *et al* 1995a, VanHeek *et al* 1997). For example, studies in mice have shown that high fat diets induce an increase in leptin levels, associated with the development of obesity. The mice, however, become obese without decreasing caloric intake (Frederich *et al* 1995a). These studies further suggest that a high content of dietary fat changes the ‘set-point’ for body weight, partly by limiting the actions of leptin. It has also been demonstrated that diet-induced obese mice exhibit resistance to peripheral leptin but

retain sensitivity to centrally administered leptin (VanHeek *et al* 1997). This finding is consistent with the observation that leptin transport into the CSF is saturable (Caro *et al* 1996). However, in contrast to the above findings, it has been shown that in diet-induced obese rats, the response to central leptin administration is attenuated compared to control animals (Widdowson *et al* 1997). The mechanisms responsible for diet-induced leptin resistance remain unknown. It has been suggested that dietary weight gain might impair the satiety signal due to either reduced receptor sensitivity, occupation of receptors by endogenous leptin molecules (Widdowson *et al* 1997), or interference with the leptin post receptor signalling pathways (Frederich *et al* 1995a). In addition, an increase in fat may stimulate hypothalamic centres involved in appetite regulation that are downstream of the initial leptin target (Frederich *et al* 1995a). Further studies are required in this area.

Overall calorie intake was slightly decreased by leptin treatment in animals offered a choice of foods, but this decrease did not reach statistical significance. Furthermore, the proportion of laboratory diet and mealworms consumed differed significantly between the leptin and PBS treated animals. Following the first injection of leptin on *day 7*, animals offered a choice of foods reduced their 24-hour intake of the laboratory diet compared to PBS injected controls. This decrease was counteracted by a non-significant increase in the intake of the preferred high calorie diet of mealworms. Consequently, an increased proportion of the higher fat, calorie dense mealworms was consumed relative to the PBS injected controls. The failure of leptin treatment to decrease the intake of mealworms is consistent with the hypothesis that

leptin has a limited ability to restrain intake of highly palatable diets (Widdowson *et al* 1997).

The effect of leptin on body weight is not due solely to a reduction in food intake, but also to an overall increase in energy expenditure. Single doses of leptin administered both centrally and peripherally increase  $VO_2$  in the *ob/ob* mouse (Halaas *et al* 1997, Hwa *et al* 1997, Pelleymounter *et al* 1995). In normal mice, leptin prevents the decrease in energy expenditure during the nadir of the diurnal cycle and also prevents the decrease in energy expenditure that would normally occur with weight loss (Hwa *et al* 1997). In *S. crassicaudata* fed laboratory diet alone, body temperature and  $VO_2$  were similar in both PBS and leptin treated animals, despite a significant fall in body weight of the leptin treated animals. This suggests an overall increase in energy expenditure, and our results are therefore consistent with previous observations (Halaas *et al* 1995, Hwa *et al* 1997, Pelleymounter *et al* 1995). Among the animals offered a choice of foods,  $VO_2$  was observed to be lower in the leptin treated animals compared to PBS treated controls at *day 15*. This finding can be attributed to an increase in  $VO_2$  in PBS treated animals as opposed to a decline in leptin treated animals. The absolute  $VO_2$  in the leptin treated animals did not change from baseline to *day 15*. The increased weight in the PBS treated animals was presumably associated with an increased energy expenditure. Why this same effect was not observed in the leptin treated animals is unknown.

Recent studies in the dasyurid *Sminthopsis macroura* have demonstrated that peripheral administration of murine recombinant leptin increases energy expenditure

by inhibiting daily torpor (Geiser *et al* 1998). Leptin 5mg/kg for 4 days halved the duration of torpor bouts and increased the average minimum body temperature reached during torpor by 4.5 °C. Interestingly, leptin did not increase metabolic rates of *S. macroura* under thermoneutral conditions (Geiser *et al* 1998). This data is consistent with our findings, and with findings in rodents demonstrating that leptin does not activate thermogenesis under thermoneutral conditions (Schmidt *et al* 1997, Stehling *et al* 1997).

In addition to effects on energy expenditure, leptin administration has also been shown to affect nutrient partitioning. In *ob/ob* mice, leptin causes a decrease in RQ, indicating an increase in fat oxidation (Hwa *et al* 1997). Similarly in lean rats, centrally administered leptin (10 µg) induces a decrease in RQ (Wang *et al* 1999). In *S. crassicaudata*, no acute effect of leptin (2.5mg/kg) on RQ was found, however in animals fed laboratory diet only, RQ increased in the leptin treated animals, indicating a change from fat to carbohydrate utilisation. RQ however was only measured after 6 and 9 days of leptin treatment. An initial decrease in RQ may have been observed if measurements were taken closer to the onset of leptin treatment, as the animals oxidised fat in response to leptin. Substrate utilisation switches from fat to carbohydrate as fat stores become depleted, and RQ increases as a consequence. Despite the consumption of a higher fat diet by the leptin treated animals which received both laboratory diet and mealworms, there was no change in RQ. This suggests that fat was stored rather than oxidised.

In *S. crassicaudata*, plasma glucose levels failed to change significantly in response to leptin. This finding is consistent with previous studies. Although in the hyperinsulinemic and hyperglycaemic *ob/ob* mouse, leptin administration decreases serum glucose and insulin, lean controls show no change in glucose or insulin with leptin administration (Pelleymounter *et al* 1995). In addition, Kamohara *et al* (1997), found that neither central or peripheral leptin infusion, affected plasma glucose or insulin levels in wild type lean mice. The above study did however show acute effects of leptin infusion on glucose metabolism; a 5 hour infusion increased glucose turnover and glucose uptake but decreasing hepatic glycogen content (Kamohara *et al* 1997).

Whether a higher dose of leptin may have overcome the effect of the mealworm diet used in this study was not addressed. The dose used, and the frequency of leptin administration, was based on published studies in other species and the results of several preliminary studies. The dose chosen was the minimum dose that reproducibly produced a decrease in body weight and food intake in this animal. The object of the study was to determine whether this dose of leptin would also prevent an increase in adiposity when the animals were fed a preferred diet which contained more calories and increased fat and protein as compared to carbohydrate. The issue of whether a supra-pharmacological dose may have had an effect was not addressed. Additional studies are also needed to determine the effect of specific macronutrients and how these interact with caloric content. A further limitation of this study is the absence of a pair fed control group of animals to account for changes in weight on the measured parameters, and this should be included in future study designs. Nevertheless, the observations obtained are consistent with those in other species.

It can be concluded that leptin has effects on food intake, energy expenditure and fat mass in *S. crassicaudata*. Furthermore, *S. crassicaudata*, like rodents, exhibits diet-induced resistance to the actions of leptin; leptin fails to decrease the intake of a highly palatable, calorie dense, high fat diet, or to induce either weight loss or prevent weight gain when this diet is offered. These findings provide further evidence that the actions of leptin are highly conserved.

## CHAPTER 8

**GLUCOPRIVATION IN *S. CRASSICAUDATA* AND ITS EFFECTS ON APPETITE****8.1 SUMMARY**

The role of metabolic fuel availability, in particular glucose, on the regulation of food intake in *S. crassicaudata*, and other marsupials, is unknown. The aim of this study was to determine the effect of insulin induced hypoglycaemia and metabolic blockade of glucose utilisation on food intake in *S. crassicaudata*. It was shown that neither insulin-induced hypoglycaemia (insulin at 0.1, 0.25, and 0.5 U/kg), nor metabolic blockade of glucose utilisation with 2-deoxy-D-glucose (2-DG) (2-DG at 100, 200, 500, and 750 mg/kg) affect food intake, despite inducing a decrease and increase in blood glucose concentrations respectively. Photoperiod had no effect on the food intake of *S. crassicaudata* in response to insulin (0.5 U/kg) or 2-DG (500 mg/kg) administration. 2-DG also had no effect on food intake when animals were offered the highly palatable, and preferred diet of mealworms. In addition, the increase in food intake after a 24-hour fast was not modified by 2-DG administration. These data indicate that glucose availability is not of primary importance to the regulation of food intake in *S. crassicaudata*.

## 8.2 INTRODUCTION

There is strong evidence that glucoprivation is an important stimulus of food intake in many mammals, including ruminants, rats, rabbits and monkeys (Houpt 1974, Houpt & Hance 1971, Smith & Epstein 1969). As the result of hypoglycaemia, insulin produces cerebral glucoprivation, increasing glucose utilisation in the peripheral tissues but decreasing brain glucose utilisation (Rezek 1976). 2-deoxy-D-glucose (2-DG) is a competitive inhibitor of intracellular glucose metabolism in the brain and periphery. By competing with glucose-6-phosphate (the phosphorylation product of glucose) for phosphoglucose isomerase, 2-DG results in the accumulation of glucose-6-phosphate and direct inhibition of glucose metabolism (Brown 1962, Wick *et al* 1957). Cellular glucose utilisation can therefore be reduced either directly using 2-DG, or indirectly using insulin.

In some animals the effects of insulin-induced hypoglycaemia and 2-DG on food intake differ. For example, in golden hamsters (*Mesocricetus auratus*) (Ritter & Balch 1978, Rowland 1978, Sclafani & Eisenstadt 1980), gerbils (*Meriones unguiculatus*) (Rowland 1978), and deermice (*Peromyscus maniculatus*) (Rowland *et al* 1985), insulin-induced hypoglycaemia increases food intake, while 2-DG does not. In contrast, in birds such as the white crowned sparrow (*Zonotrichia leucophrys gambelii*) and chicken (*Gallus domesticus*), neither insulin-induced hypoglycaemia, nor 2-DG increase food intake (Boswell *et al* 1995, Hatfield & Smith 1972, Smith & Bright-Taylor 1974). A summary of these previous studies is provided in Table 8.1.



|                              | Insulin | 2-DG |                                                        |
|------------------------------|---------|------|--------------------------------------------------------|
| Humans                       | +       | +    | Silverstone & Besser 1971,<br>Thompson & Campbell 1977 |
| Monkeys                      | +       | +    | Smith & Epstein 1969                                   |
| Rats                         | +       | +    | Smith & Epstein 1969, Brandes<br>1977                  |
| Rabbits                      | +       | +    | Houpt & Hance 1971                                     |
| Ruminants (sheep<br>& goats) | +       | +    | Houpt 1974                                             |
| Domestic cats                | ?       | -    | Jalowiec <i>et al</i> 1973                             |
| Golden hamsters              | +       | +    | Rowland 1983, Silverman<br>1978, DiBattista 1983       |
| Gerbils                      | +       | -    | Rowland 1978                                           |
| Deermice                     | +       | -    | Rowland <i>et al</i> 1985                              |
| Marsupials                   | ?       | ?    |                                                        |
| White-crowned<br>sparrows    | -       | -    | Boswell <i>et al</i> 1995                              |
| Chickens                     | -       | -    | Hatfield & Smith 1972, Smith<br>& Bright-Taylor 1974   |

**Table 8.1:** A summary of the effects of insulin induced hypoglycaemia and 2-DG on food intake in a range of animals. + indicates that an increase in food intake was observed; - indicates that no increase in food intake was observed; ? indicates a currently unknown effect.

The aetiology of the discrepant responses between animals in their feeding responses to insulin and 2-DG is uncertain.

The impact of metabolic fuel availability on the regulation of food intake in marsupials has not been studied. In the marsupial *S. crassicaudata*, 24-hour food deprivation is associated with reductions in body weight, tail width and blood glucose, and a 50-100% increase in food intake when food is reintroduced after this time (see chapter 4). The factors responsible for this increase in food intake are unknown. It is hypothesised that a decrease in glucose availability triggers the increased food intake in *S. crassicaudata*. Therefore, the aim of this study was to determine the effect of insulin-induced hypoglycaemia and 2-DG on food intake in the marsupial *S. crassicaudata*.

## **8.3 MATERIALS AND METHODS**

### **8.3.1 *Experimental animals, diet and drugs***

Adult male *S. crassicaudata* housed under either a long-day (LD; 16-h light, 8-h dark) or a short-day (SD; 8-h light, 16-h dark) light regime; lights turned off at 16:30h, were studied. Laboratory diet was provided *ad libitum* except where stated. Mealworms were provided *ad libitum* where stated. Food intake was expressed as kcal.

Insulin and 2-deoxy-D-glucose (2-DG) were prepared and administered as stated in section 3.5.2.

### 8.3.2 *Experimental protocols*

#### Study 1: Effect of insulin and 2-DG on blood glucose concentrations

To determine the effect of insulin and 2-DG on blood glucose concentrations, LD housed *S. crassicaudata*, permitted *ad libitum* access to standard laboratory diet, were injected with either insulin (0.1, 0.5, 1.0 U/kg) or 2-DG (100, 200, 500, 750 mg/kg) at the onset of the dark phase ( $n = 3$  per group). Blood was obtained by puncturing the orbital sinus (see section 3.4), and the glucose concentration immediately measured (see section 3.4.1). Blood glucose concentrations were determined at 0, 30 and 45 minutes in animals injected with insulin, and at 0 and 60 minutes in animals injected with 2-DG. Sampling intervals were based on studies in other mammals demonstrating the maximum effect of 2-DG on blood glucose is at approximately 1-hour, and insulin at approximately 0.5-1 hours post-administration (Brandes 1977, Houpt 1974, Ritter & Balch 1978, Rowland 1978).

#### Study 2: Effect of a reduction in cellular glucose utilisation induced by insulin-induced hypoglycaemia or 2-DG on food intake.

To determine the effect of a reduction in cellular glucose utilisation by insulin-induced hypoglycaemia or 2-DG on food intake, LD housed *S. crassicaudata* were divided into groups of similar body weight ( $n = 7-8$  per group). Thirty minutes prior to the onset of the dark phase animals were weighed and all food removed. At lights off, animals were injected with 2-DG (100, 200, 500, 750 mg/kg), insulin (0.1, 0.25, 0.5

U/kg) or 0.9% saline, and a pre-weighed amount of laboratory diet placed in each cage. For each animal, subsequent food intake was recorded at 0.5, 1, 2, 4 and 24 hours.

Study 3: *Effect of a reduction in cellular glucose utilisation by insulin-induced hypoglycaemia or 2-DG on food intake, in response to photoperiod.*

To determine if the effect of a reduction in cellular glucose utilisation by insulin-induced hypoglycaemia or 2-DG on food intake is modified by photoperiod, SD housed *S. crassicaudata* were divided into groups of similar body weight ( $n = 10$ -12 per group). The experiment was performed as in study 2, however only one dose of insulin (0.5 U/kg) and 2-DG (500 mg/kg) was used.

Study 4: *Effect of a reduction in cellular glucose utilisation by 2-DG on food intake in animals with a strong desire to eat.*

To determine the effect of a reduction in cellular glucose utilisation by 2-DG on food intake in animals with a strong desire to eat, the protocol was as for study 2 ( $n = 7$  per group). The experiment, however, was carried out in animals i) re-fed with laboratory diet following a 24-hour fast (see chapter 4), and ii) re-fed with a preferred high calorie diet of mealworms (see chapter 11). 2-DG was injected at doses of 100, 200, 500 and 750 mg/kg.

### **8.3.3      *Statistical analyses***

Data analyses were carried out as stated in section 3.9.

## 8.4 RESULTS

### 8.4.1 Study 1: Effect of insulin and 2-DG on blood glucose

In response to insulin at 0.1 U/kg, blood glucose decreased from a mean baseline of  $4.5 \pm 0.3$  mmol/l to  $3.2 \pm 1.2$  mmol/l at 30 minutes and was  $3.7 \pm 0.2$  mmol/l at 45 minutes. In response to insulin at a dose of 0.5 U/kg blood glucose decreased to  $2.2 \pm 0.3$  mmol/l at 30 minutes and was  $2.7 \pm 0.7$  mmol/l at 45, and in response to 1.0 U/kg insulin blood glucose decreased to  $1.9 \pm 0.5$  mmol/l at 30 minutes and  $1.4 \pm 0.1$  mmol/l at 45 minutes, Fig 8.1A.

In response to 2-DG, blood glucose increased within 60 minutes from a mean baseline of  $2.9 \pm 0.2$  mmol/l to  $3.1 \pm 0.8$  mmol/l at a dose of 100 mg/kg, to  $3.5 \pm 0.2$  mmol/l at a dose of 200 mg/kg, to  $6.0 \pm 1.6$  mmol/l at a dose of 500 mg/kg and to  $10.5 \pm 0.1$  mmol/l at a dose of 750 mg/kg, Fig 8.1B.

### 8.4.2 Study 2: Effect of insulin-induced hypoglycaemia and 2-DG on food intake

Baseline body weights did not differ between the groups for any experiment.

Neither insulin at 0.1 U/kg (drug  $F_{1,79} = 1.00$ ,  $P = 0.33$ , drug x time  $F_{4,79} = 1.01$ ,  $P = 0.41$ ), 0.25 U/kg (drug  $F_{1,69} = 0.21$ ,  $P = 0.66$ , drug x time  $F_{4,69} = 0.21$ ,  $P = 0.93$ ) or 0.5 U/kg (drug  $F_{1,79} = 1.00$ ,  $P = 0.33$ , drug x time  $F_{4,79} = 1.00$ ,  $P = 0.41$ ), Fig 8.2A, nor 2-DG at 100 mg/kg (drug  $F_{1,69} = 0.18$ ,  $P = 0.68$ , drug x time  $F_{4,69} = 1.2$ ,  $P = 0.32$ ), 200 mg/kg (drug  $F_{1,69} = 0.22$ ,  $P = 0.65$ , drug x time  $F_{4,69} = 0.80$ ,  $P = 0.53$ ), 500 mg/kg (drug  $F_{1,69} = 0.21$ ,  $P = 0.66$ , drug x time  $F_{4,69} = 2.63$ ,  $P = 0.05$ ) or 750 mg/kg

(drug  $F_{1,69} = 0.01$ ,  $P = 0.91$ , drug x time  $F_{4,69} = 0.01$ ,  $P = 0.99$ ), Fig 8.2B, had any effect on food intake when compared to saline treated controls.

#### 8.4.3 Study 3: Effect of insulin-induced hypoglycaemia and 2-DG on food intake in response to photoperiod.

Baseline body weights did not differ between the groups for any experiment.

Neither insulin at 0.5 U/kg (drug  $F_{1,119} = 1.43$ ,  $P = 0.24$ , drug x time  $F_{4,119} = 1.43$ ,  $P = 0.23$ ), Fig 8.3A, nor 2-DG at 500 mg/kg (drug  $F_{1,104} = 0.99$ ,  $P = 0.33$ , drug x time  $F_{4,104} = 0.99$ ,  $P = 0.42$ ), Fig 8.3B, had an effect on food intake compared to saline treated controls in short-day housed animals.

#### 8.4.4 Study 4: Effect of 2-DG on food intake in response to a preferred food.

Baseline body weights did not differ between the groups for any experiment.

i) In animals that had been food deprived for 24 hours, 2-DG had no effect on food intake at 100 mg/kg (drug  $F_{1,69} = 0.95$ ,  $P = 0.35$ , drug x time  $F_{4,69} = 1.81$ ,  $P = 0.14$ ), 200 mg/kg (drug  $F_{1,69} < 0.0001$ ,  $P = 0.99$ , drug x time  $F_{4,69} = 1.24$ ,  $P = 0.31$ ) or 500 mg/kg (drug  $F_{1,69} = 1.36$ ,  $P = 0.27$ , drug x time  $F_{4,69} = 0.62$ ,  $P = 0.65$ ). At 750 mg/kg, 2-DG administration resulted in a decrease in food intake as compared to saline treated controls (drug  $F_{1,69} = 5.35$ ,  $P = 0.04$ , drug x time  $F_{4,69} = 0.39$ ,  $P = 0.81$ ), Fig 8.4A

ii) In animals re-fed with the preferred high calorie diet of mealworms, 2-DG had no effect on food intake as compared to the saline treated controls in either the 100

mg/kg (drug  $F_{1,69} = 0.99$ ,  $P = 0.34$ , drug x time  $F_{4,69} = 0.99$ ,  $P = 0.42$ ) or the 200 mg/kg (drug  $F_{1,69} = 1.01$ ,  $P = 0.33$ , drug x time  $F_{4,69} = 1.01$ ,  $P = 0.41$ ) groups. A reduction in food intake as compared to saline treated controls was observed in the 500 mg/kg (drug  $F_{1,69} = 3.31$ ,  $P = 0.09$ , drug x time  $F_{4,69} = 5.27$ ,  $P = 0.001$ ) and the 750 mg/kg (drug  $F_{1,69} = 7.99$ ,  $P = 0.02$ , drug x time  $F_{4,69} = 0.53$ ,  $P = 0.71$ ) groups, Fig 8.4B

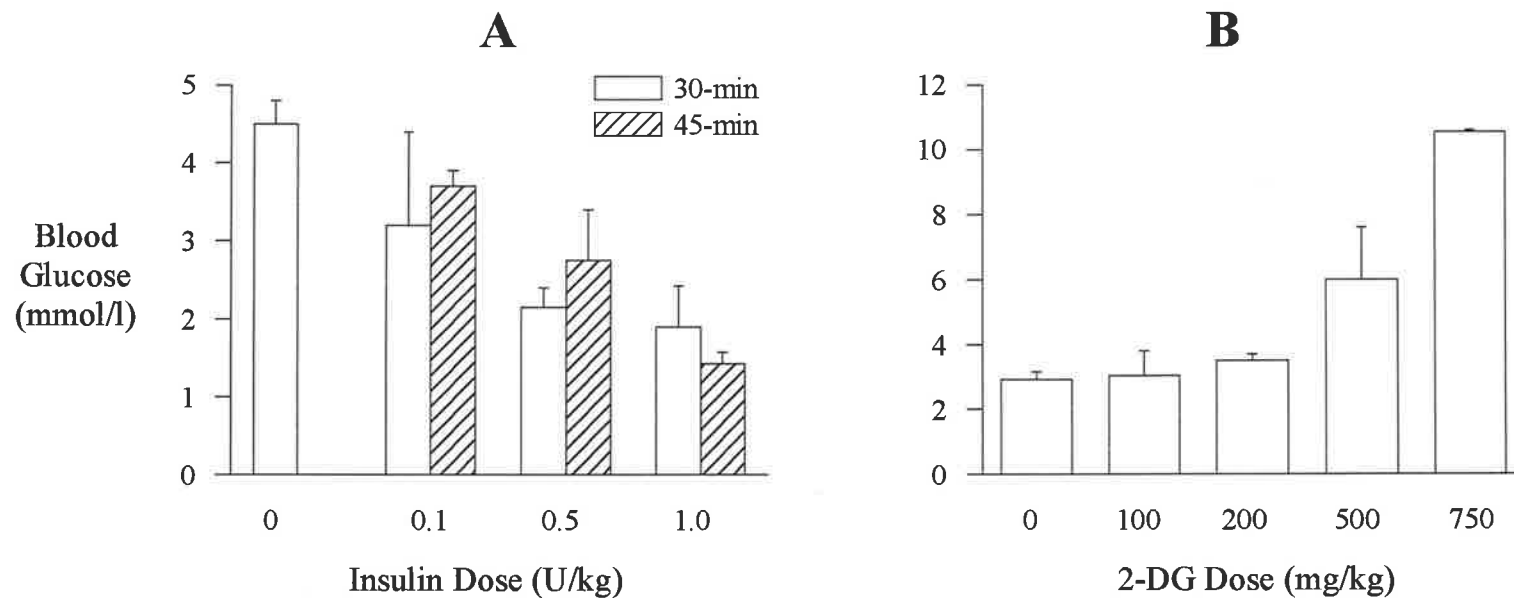


**Figure 8.1:** Effect of (A) IP insulin administration (0.1, 0.5 and 1.0 U/kg) on blood glucose concentrations at baseline (0 U/kg) and at 30 and 45 minutes post-injection, and (B) IP 2-DG administration (0, 100, 200, 500, and 750 mg/kg) on blood glucose concentration 60 minutes post-injection, on male *S. crassicaudata* housed under a LD photoperiod (16-h light, 8-h dark). Insulin and 2-DG induce a decrease and an increase in blood glucose concentrations respectively. Mean  $\pm$  SE,  $n = 3$  per group.



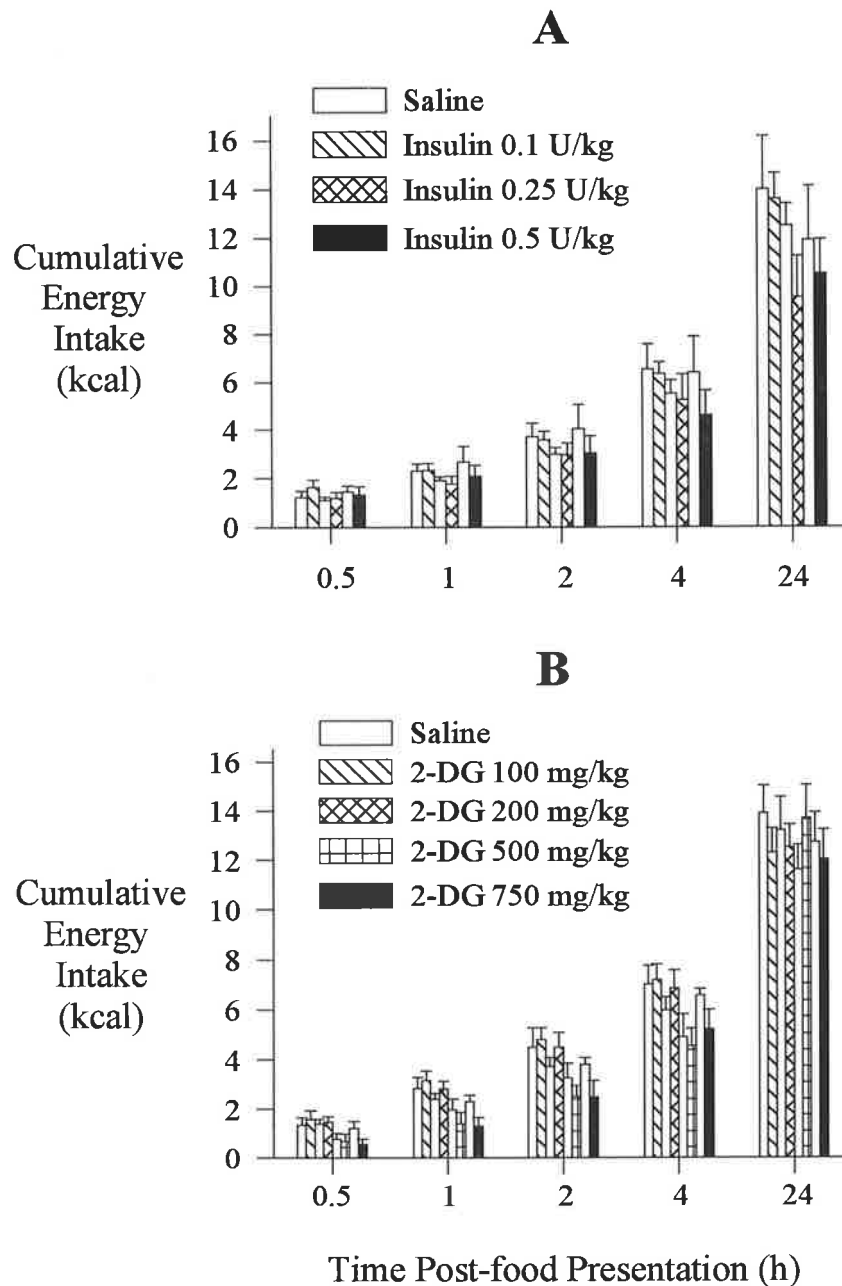


**Figure 8.2:** Effect of (A) IP insulin administration (0.1, 0.25, and 0.5 U/kg), and (B) IP 2-DG administration (100, 200, 500, and 750 mg/kg) on subsequent 24-h food intake in male *S. crassicaudata* housed under a LD photoperiod (16-h light, 8-h dark). Neither insulin nor 2-DG affected food intake when compared to the saline treated controls. Mean  $\pm$  SE,  $n = 7-8$  per group.

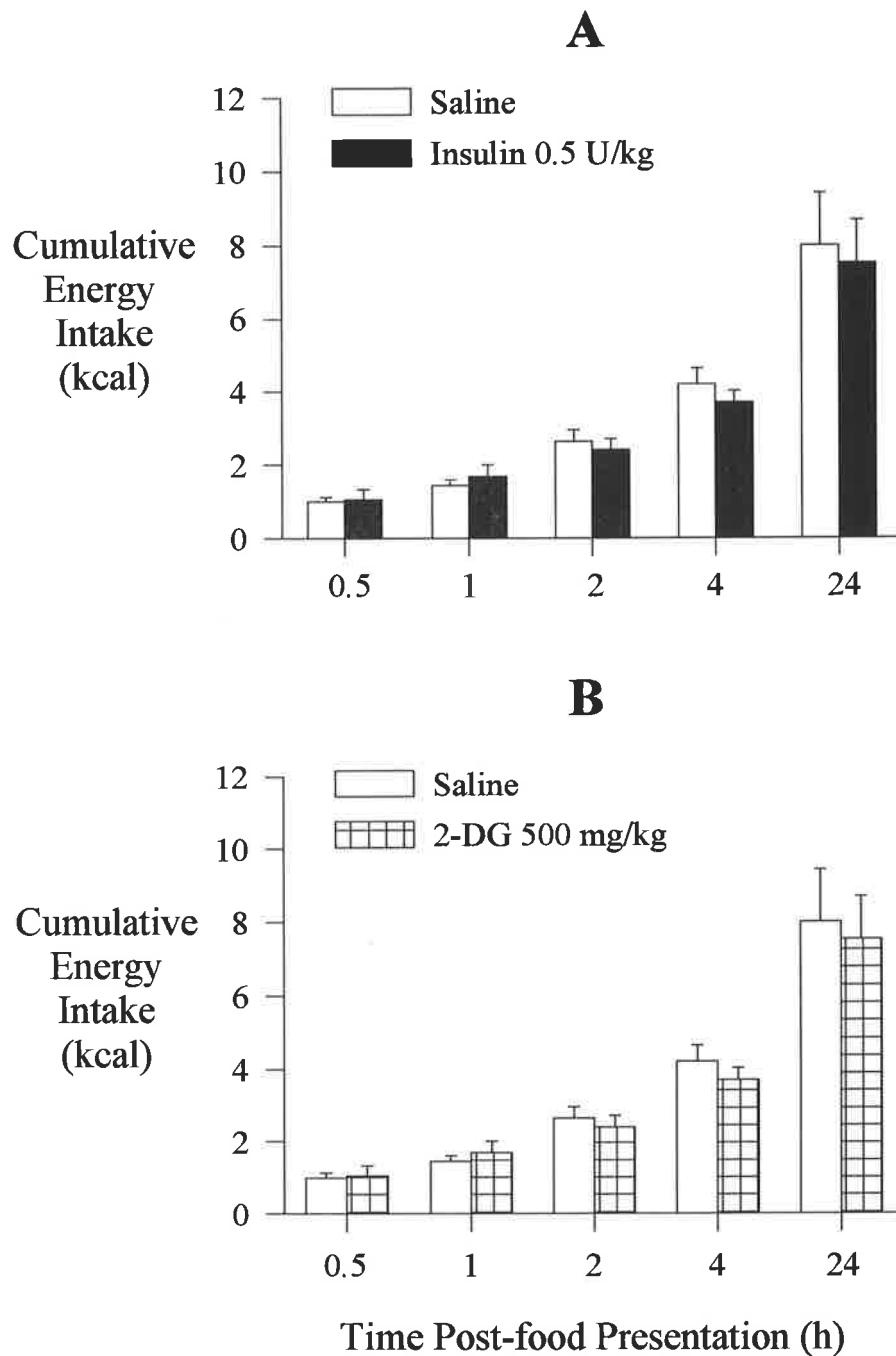


**Figure 8.3:** Effect of (A) IP insulin administration (0.5 U/kg) and (B) IP 2-DG administration (500 mg/kg) on subsequent 24-h food intake in male *S. crassicaudata* housed under a SD photoperiod (8h-light, 16-h dark). Neither insulin nor 2-DG affected food intake when compared to the saline treated controls. Mean  $\pm$  SE,  $n = 10-12$  per group.

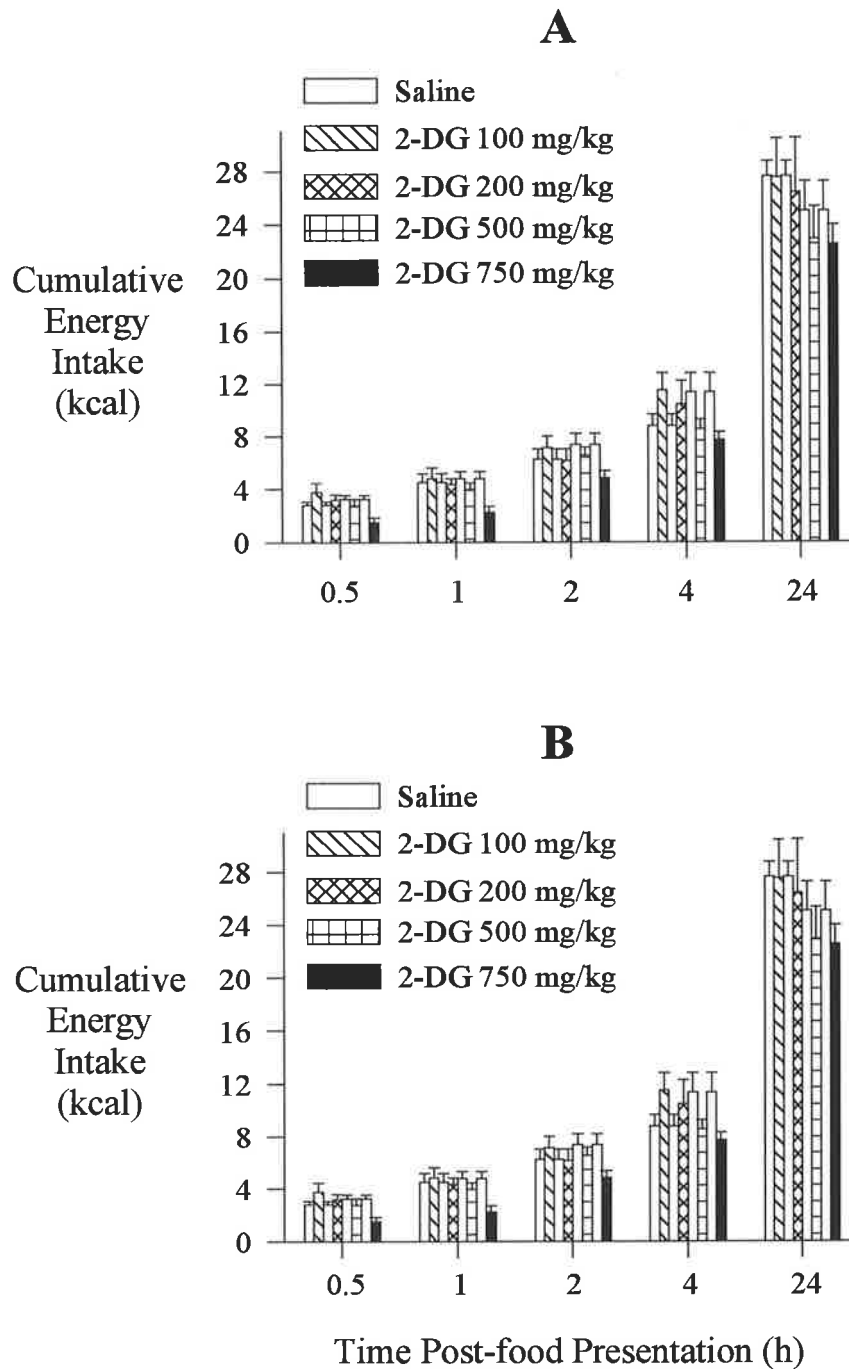


**Figure 8.4:** Effect of IP 2-DG administration (100, 200, 500, and 750 mg/kg) on subsequent 24-h food intake in male *S. crassicaudata* housed under a LD photoperiod (16:8h, light:dark), that have (A) been deprived of food for 24-h, and (B) been re-fed with the preferred high calorie diet of mealworms. Mean  $\pm$  SE,  $n = 7$  per group.

## 8.5 DISCUSSION

The absence of an increase in food intake in response to either insulin or 2-DG, in any of the experimental paradigms tested, suggests that neither the blood glucose concentration nor glucose utilisation are important determinants of food intake in *S. crassicaudata*.

The failure of *S. crassicaudata* to respond to glucoprivation by increasing food intake contrasts with many other mammals, but is comparable to observations in birds; neither the white-crowned sparrow (Boswell *et al* 1995) nor the chicken (Hatfield & Smith 1972, Smith & Bright-Taylor 1974) increase their food intake in response to a decrease in glucose utilisation. However, even in mammals, the relationship between glucoprivation and food intake is inconsistent (Brandes 1977, Houpt & Hance 1971, Smith & Epstein 1969). While rats increase their food intake in response to food deprivation, insulin hypoglycaemia and 2-DG, in deermice (*Peromyscus maniculatus*) and gerbils (*Meriones unguiculatus*) food deprivation stimulates feeding, but not 2-DG; only a modest increase in food intake occurs in response to insulin induced hypoglycaemia (Rowland 1978, Rowland *et al* 1985). The Syrian hamster (*Mesocricetus auratus*) does not increase food intake in response to either food deprivation or 2-DG, and only variably in response to short acting insulin (Ritter & Balch 1978). Interestingly, after food deprivation, the Syrian hamster is more sensitive to the hyperphagic effects of insulin (DiBattista 1983).

Photoperiod may influence the response to glucoprivation. The Siberian hamster (*Phodopus sungorus sungorus*) does not exhibit post fast hyperphagia, and those animals housed on short photoperiods eat less than those housed on long photoperiods. This animal responds to both 2-DG and short acting insulin with an increase in food intake, but only in those animals housed under long photoperiods (Bartness *et al* 1995). In *S. crassicaudata*, no effect of photoperiod on the response to insulin or 2-DG was found.

As in rats and gerbils, *S. crassicaudata* increase their food intake after fasting (see chapter 4). However, fasted animals failed to respond to 2-DG by increasing food intake. While it is unlikely that the observed increase in food intake following 24-hour food deprivation in *S. crassicaudata* is directly attributable to the fall in plasma glucose levels, it may be related to changes in other plasma nutrients (for example free fatty acids), a decrease in fat mass or both. The effect of a combination of food deprivation and insulin-induced hypoglycaemia was not examined in this study because of concern for the welfare of the animals. Plasma glucose concentration falls significantly with fasting (see chapter 4), and the animals are sensitive to the hypoglycaemic effects of insulin.

In rats, the effects of insulin and 2-DG (Brandes 1977, Houpt & Hance 1971) on food intake are dependent on the palatability of the diet. However, hamsters fail to increase food intake in response to 2-DG even when offered a highly palatable diet (Sclafani & Eisenstadt 1980). *S. crassicaudata* prefer an insectivorous diet to their usual laboratory diet (see chapter 11) and for this reason the feeding response to 2-DG

when animals were re-fed with a preferred diet of mealworms was examined. As observed in animals fed laboratory diet, intake of mealworms did not increase after 2-DG.

In 24-hour food deprived *S. crassicaudata* there was a decrease in food intake when 2-DG was given in a dose of 750 mg/kg; this was also the case in animals given 2-DG at 500 and 750 mg/kg and subsequently re-fed with mealworms. Previous studies have reported a decrease in food intake after administration of insulin or 2-DG. These decreases in food intake have been previously attributed to stupor and ataxia produced by high doses of 2-DG, which, in some instances, has been shown to be diet dependent (Rowland 1978, Sclafani & Eisenstadt 1980, Vasselli & Sclafani 1976). Presumably, the decreases in food intake observed in *S. crassicaudata* in response to the higher doses of 2-DG occurred because the animals were unwell. Although no direct observations or measurements were made, *S. crassicaudata* receiving higher doses of 2-DG did in some instances appear to be less active and co-ordinated.

There are several potential limitations in the use of insulin and 2-DG to evaluate glucostatic feeding mechanisms. For example, it has been suggested that cytoglucopenia is only one of the complex metabolic shortages induced by insulin, and that since metabolites other than glucose can suppress the hyperglycaemic response to 2-DG, cytoglucopenia *per se* may not be sufficient to trigger the feeding response (Friedman & Stricker 1976). Furthermore, it has been shown in rats that it is not the decline in blood glucose *per se* that predicts the onset of feeding or overeating, but rather the timing of neurological dysfunction (Brandes 1977). Even in species with a

demonstrated glucoprivic feeding system such as the rat, this system may function as an emergency back up, rather than one participating in the daily regulation of food intake and body weight (Epstein *et al* 1975). Nevertheless, the current data, taken together with that of others, suggest that glucoprivation is not a highly conserved mechanism for signalling the need to increase food intake.

Many animals decrease energy expenditure and basal metabolic rate to conserve energy and preserve body composition, rather than increasing food intake. Increases in food intake may be driven primarily by signals reflecting alterations in body composition, or other metabolic signals such as free fatty acids reflecting changes in energy balance, or by photoperiodic or temperature related cues. These mechanisms in general could be potentially more important than alterations in plasma glucose or glucose utilisation, as may be the case with *S. crassicaudata*.

## CHAPTER 9

**FATTY ACIDS AND FOOD INTAKE IN *S. CRASSICAUDATA*****9.1 SUMMARY**

There is evidence that the oxidation of metabolic fuels may provide post-absorptive satiety signals, which are important in the regulation of appetite. In rats and mice, inhibition of fatty acid oxidation, using mercaptoacetate (MA) or methyl palmoxirate, stimulates food intake in rats and mice. In *S. crassicaudata* food intake does not increase in response to glucoprivation (see chapter 8), suggesting that other metabolic fuels, such as free fatty acids (FFA), may play an important role in appetite regulation in this species. The aim of this study was to determine the effect of MA on food intake in *S. crassicaudata*.

Male *S. crassicaudata* housed under a long-day photoperiod (16-h light, 8-h dark) were injected with either saline or MA (100, 200, 400, 600, 800  $\mu\text{mol/kg}$ ) during the light phase (08:30h). Blood was sampled immediately prior to injection, and at 1- and 6-hours post-injection for measurement of plasma FFA. Subsequently, male *S. crassicaudata* were divided into 2 groups ( $n = 8-10$ ). One group received saline and the other intraperitoneal MA (100, 200, 400, 600, 800  $\mu\text{mol/kg}$ ) at the onset of the dark phase (14:30h), or during the light phase (08:30h). Food intake was subsequently measured at 0.5, 1, 2, 4, 6, and 24 hours.



Following MA administration, plasma FFA levels increased in a dose dependent manner ( $P < 0.05$ ). At the onset of the dark phase, MA 400 and 600  $\mu\text{mol/kg}$  had no effect on food intake, but MA 100 and 200  $\mu\text{mol/kg}$  induced a 26% decrease ( $P = 0.01$ ) and 36% increase ( $P < 0.0001$ ) in food intake respectively, at the 24-hour time point only. The highest dose (800  $\mu\text{mol/kg}$ ) decreased food intake at all time points ( $P = 0.04$ ). During the light phase, MA (200, 400, 600, 800  $\mu\text{mol/kg}$ ) had no effect on food intake, whereas MA 100  $\mu\text{mol/kg}$  decreased food intake by 20% at 6 hours and by 29% at 24 hours ( $P = 0.03$ ).

These data demonstrate that in *S. crassicaudata*: i) MA increases plasma FFA concentration in a dose-dependent manner, as a result of decreased fatty acid oxidation, and ii) inhibition of fatty acid oxidation by MA does not acutely increase the intake of standard laboratory diet under non-fasted conditions.

## 9.2 INTRODUCTION

Changes in the plasma levels, and/or utilisation of metabolic fuels such as glucose and free fatty acids, affect food intake in a range of species (Friedman *et al* 1986, Friedman & Stricker 1976). The impact of alterations in storage and oxidation of fat on food intake has previously been examined using agents that either inhibit fatty acid oxidation, or by intravenous infusion of lipid (Booth & Campbell 1975, Friedman & Tordoff 1987). Fatty acid oxidation can be inhibited either indirectly by reducing the availability of fatty acids (eg. using antilipolytic agents), or directly by decreasing fatty

acid oxidation. Methyl palmoxirate and etomoxir (the oxirane carboxylates) reduce fatty acid oxidation by irreversibly inhibiting transport by carnitine palmitoyl transferase I (CPT I), of long-chain fatty acids into mitochondria (McGarry & Foster 1980, Tutwiler *et al* 1985, Wolf 1990), whereas mercaptoacetate (MA) suppresses fatty acid oxidation by inhibiting the acyl-coA dehydrogenases that catalyse mitochondrial  $\beta$ -oxidation (Bauche *et al* 1983). Impairment of  $\beta$ -oxidation decreases in mitochondrial production of reducing equivalents (Bremer & Osmundsen 1984), which may influence food intake (Scharrer & Langhans 1986).

In rats and mice, inhibition of fatty acid oxidation increases food intake in rats and mice (DelPrete *et al* 1998, Friedman *et al* 1999, Langhans & Scharrer 1987), while increased oxidation of fatty acids is associated with a decrease in food intake (Langhans *et al* 1985a,b). Inhibition of fatty acid oxidation increases plasma FFA levels and reduces the end products of fatty acid oxidation (*ie.* ketone bodies) (Young *et al* 1995). A decrease in fatty acid oxidation is compensated for by an increase in glucose oxidation and consequently plasma glucose and liver glycogen levels fall (Tuman *et al* 1988, Tutwiler & Dellevigne 1979). The exact mechanism by which inhibitors of fatty acid metabolism affect food intake remains to be determined. While it has been suggested that alterations in metabolite levels are responsible for the observed increases in food intake (Langhans & Scharrer 1987), a recent study indicates that decreases in liver glycogen, blood glucose and ketone levels are unlikely to be directly responsible for the observed increase in food intake following methyl palmoxirate (MP) administration in rats (Friedman *et al* 1999). (Friedman *et al* (1999) have shown that both low (1 mg/kg) and high (10 mg/kg) doses of MP decrease

plasma ketone levels to the same degree, however only the higher increases food intake. In addition, although a reduction in blood glucose stimulates eating in a range of species (Bartness *et al* 1995, Houpt 1974, Houpt & Hance 1971, Smith & Epstein 1969), injections of glucose do not prevent an increase in food intake of rats, following MP treatment (Friedman *et al* 1999). Despite suggestions that alterations in liver glycogen content affect food intake (Flatt 1987, Russek 1975), decreased liver glycogen following treatment with low doses of MP is not associated with increased food intake in rats (Friedman *et al* 1999).

In rats, the appetite response to inhibition of fatty acid oxidation using MA has been shown to vary depending on the phase of the light cycle. Fatty acid oxidation in the bright phase exceeds that of the dark phase (Booth & Campbell 1975, LeMagen *et al* 1973), and MA induced feeding has been demonstrated to be of a greater magnitude during the light as compared to the dark phase (Scharrer & Langhans 1986).

In *S. crassicaudata*, fasting increases peripheral plasma free fatty acid levels, presumably to maintain energy supply to the rest of the body. Fasting is also associated with a decrease in blood glucose levels and a subsequent increase in food intake (see chapter 4), although food intake fails to increase in response to glucoprivation (see chapter 8). These observations suggest that metabolic fuels other than glucose are important in the regulation of food intake in this species. It is hypothesised that fatty acids may play a role in feeding in *S. crassicaudata*. The aim of this study was to determine the effect of mercaptoacetate-induced inhibition of fatty acid oxidation on food intake in *S. crassicaudata*.

## 9.3 MATERIALS AND METHODS

### 9.3.1 *Experimental animals, diet and drugs*

Adult male *S. crassicaudata* housed on a long-day light regime (16-h light, 8-h dark, lights off 14:30h) were used in all experiments. Animals were fed laboratory diet *ad libitum*. Experiments were carried out in non-fasted animals. Data for food intake were expressed as kcal.

Mercaptoacetate (MA) was prepared as stated in section 3.5.3. Both saline placebo and MA were administered intraperitoneally (IP).

### 9.3.2 *Experimental protocols*

#### Study 1: *The effect of mercaptoacetate on plasma free fatty acid levels*

In order to determine the effect of mercaptoacetate on plasma free fatty acid (FFA) levels, male *S. crassicaudata* ( $n = 3$  per group) were injected IP, during the light phase (08:30h), with either 0.9% saline or MA (200, 400, 600 and 800  $\mu\text{mol/kg}$ ). Blood was sampled (see section 3.4) prior to injection, and at 1- and 6-hours post-injection, for measurement of plasma FFA (see section 3.4.2).

#### Study 2: *The effect of mercaptoacetate on food intake during the light and dark phase of the circadian cycle*

In order to determine the effect of inhibition of fatty acid oxidation on food intake, male *S. crassicaudata* were divided into 2 groups of equal body weight ( $n = 8-10$  per group). At commencement of the dark phase (14:30h), or during the light phase

(08:30h), animals were injected IP with either saline, or MA (200, 400, 600, 800  $\mu\text{mol/kg}$ ). Animals were immediately given laboratory diet *ad libitum*, and subsequent food intake recorded at 0.5, 1, 2, 4, 6, and 24 hours. Different doses of MA were given in a counterbalanced order in tests 3-4 days apart, such that no animal received more than one dose of MA within a 72-hour period.

### 9.3.3 *Statistical analyses*

Analyses were carried out as stated in section 3.9.

## 9.4 RESULTS

### 9.4.1 Study 1: Effect of mercaptoacetate on plasma free fatty acid levels

Baseline plasma FFA concentration averaged  $0.320 \pm 0.03$  mEq/l. Plasma FFA levels increased in a dose-dependent manner in response to IP MA ( $F_{4,53} = 3.14$ ,  $P = 0.049$ ), Fig 9.1. MA 100  $\mu\text{mol/kg}$  ( $P = 0.05$ ) and 200  $\mu\text{mol/kg}$  ( $P = 0.04$ ) induced a 0.31 and 0.11 mEq/l increase respectively in plasma FFA above baseline, at 1-hour after administration, Fig 9.1. MA 400  $\mu\text{mol/kg}$  induced an increase in plasma FFA concentration above baseline at both 1 and 6 hours after administration, by 0.33 ( $P = 0.03$ ) and 0.36 mEq/l ( $P = 0.045$ ) respectively. MA 600  $\mu\text{mol/kg}$  ( $P = 0.02$ ) and 800  $\mu\text{mol/kg}$  ( $P = 0.04$ ) induced an increase in plasma FFA above baseline, 6 hours after administration, by approximately 0.39 and 0.29 mEq/l respectively, Fig 9.1.

#### 9.4.2 **Study 2: Effect of mercaptoacetate on food intake during the light and dark phase**

##### *i) Onset of dark-phase*

MA 100  $\mu\text{mol/kg}$  induced a 26% decrease in food intake compared to saline controls, however, this decrease was only observed at the 24-hour time-point (drug x time  $F_{1,95} = 3.28$ ,  $P = 0.01$ ). By contrast, MA 200  $\mu\text{mol/kg}$  induced a 36% increase in food intake when compared to saline controls at the 24-hour time point ( $F_{1,95} = 14.88$ ,  $P < 0.0001$ ), Fig 9.2A. Neither MA 400  $\mu\text{mol/kg}$  ( $F_{1,107} = 0.24$ ,  $P = 0.63$ ), nor 600  $\mu\text{mol/kg}$  ( $F_{1,107} = 0.009$ ,  $P = 0.93$ ) had any effect on food intake when compared to saline at any time period. MA 800  $\mu\text{mol/kg}$  decreased food intake at all time points when compared to the saline treated controls ( $F_{1,101} = 4.9$ ,  $P = 0.04$ ), Fig 9.2A.

##### *ii) During light phase*

MA 100  $\mu\text{mol/kg}$  induced a decrease in food intake when compared to saline controls, however this decrease was only observed at the 6-hour (20% decrease) and 24-hour (29% decrease) time points (drug x time  $F_{1,95} = 2.65$ ,  $P = 0.03$ ), Fig 9.2B. In contrast, neither MA 200  $\mu\text{mol/kg}$  ( $F_{1,95} = 1.58$ ,  $P = 0.23$ ), 400  $\mu\text{mol/kg}$  ( $F_{1,107} = 1.79$ ,  $P = 0.20$ ), 600  $\mu\text{mol/kg}$  ( $F_{1,119} = 0.68$ ,  $P = 0.42$ ) nor 800  $\mu\text{mol/kg}$  ( $F_{1,119} = 0.26$ ,  $P = 0.62$ ) had any effect on food intake when compared to saline at any time period in the subsequent 24 hours after administration, Fig 9.2B.

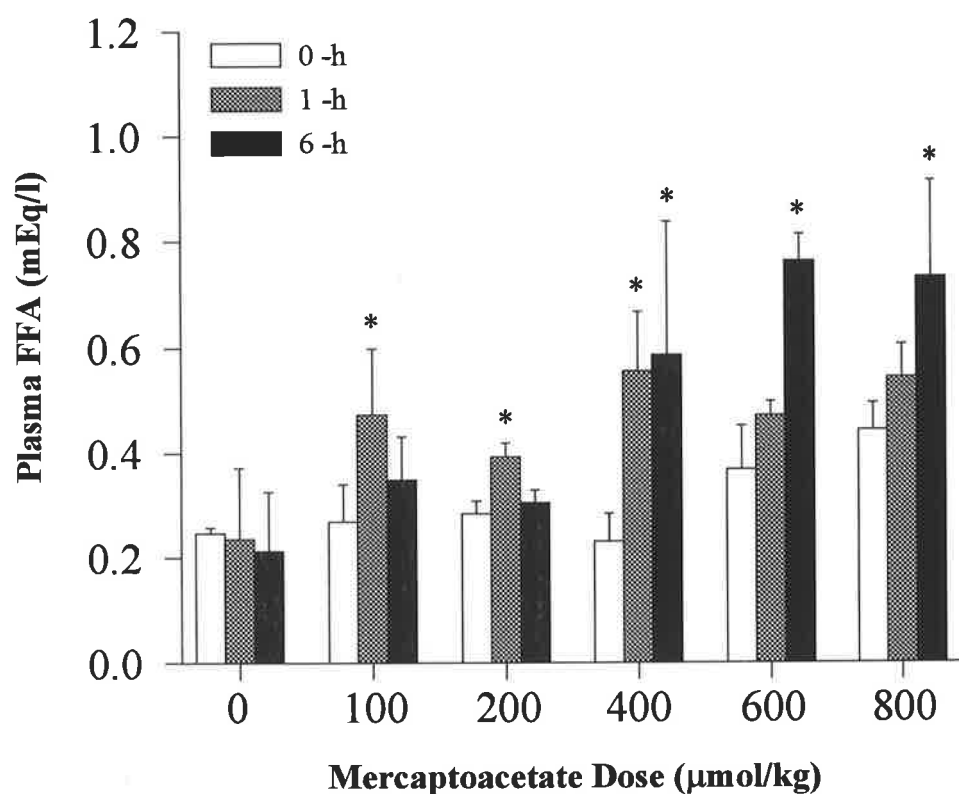


**Figure 9.1:** Effect of mercaptoacetate (0, 100, 200, 400, 600, and 800 μmol/kg IP) on plasma free fatty acid concentrations at basal (0-h) and at 1 and 6 hours post administration. Mercaptoacetate increased free fatty acids in a dose-dependent manner. Mean  $\pm$  SE, \*  $P < 0.05$  compared to baseline.

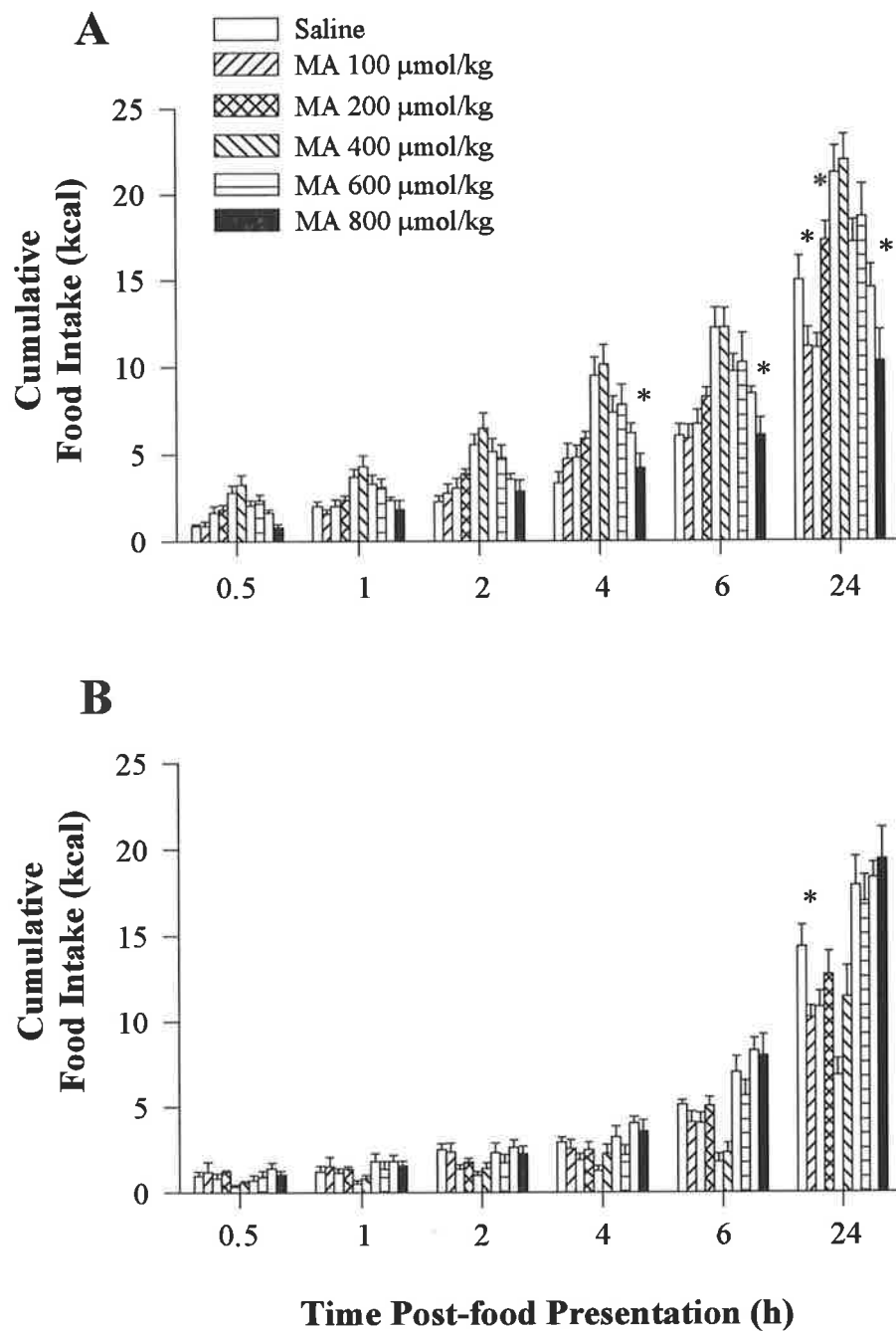


**Figure 9.2:** Effect of mercaptoacetate (100, 200, 400, 600, and 800  $\mu\text{mol/kg}$  IP) on subsequent 24-hour food intake (A) at commencement of the dark phase, and (B) during the light phase, in long-day housed (16-h light, 8-h dark) *S. crassicaudata* fed *ad libitum* with laboratory diet. Mean  $\pm$  SE, \*  $P < 0.05$  compared to saline.



## 9.5 DISCUSSION

The results of this study indicate that although fatty acid oxidation is inhibited by mercaptoacetate in *S. crassicaudata*, MA does not induce any acute alterations in food intake during either the light or dark phase of the diurnal photoperiod.

As in rats (Scharrer & Langhans 1986), Syrian hamsters (*Mesocricetus auratus*) (Schneider *et al* 1988), and cattle (Choi *et al* 1997), the intraperitoneal administration of mercaptoacetate (100, 200, 400, 600, 800  $\mu\text{mol/kg}$ ) to male *S. crassicaudata* resulted in a dose dependent increase in plasma FFA levels. Mercaptoacetate inhibits the acyl-CoA dehydrogenases located in the mitochondrial matrix, and thus impairs mitochondrial  $\beta$ -oxidation of fatty acids and ketogenesis (Bauche *et al* 1983, Foster & McGarry 1982). As a result, mitochondrial production of reducing equivalents from  $\beta$ -oxidation decreases, plasma free fatty acid levels rise, and ketone levels fall. Based on changes in plasma FFA levels, it appears that MA administration inhibits fatty acid oxidation in *S. crassicaudata*, with effects lasting for up to 6 hours, depending on dose. The stimulation of plasma FFA by MA has been shown to persist for as long as 5 hours in Syrian hamsters (Lazzarini *et al* 1988) and 6 hours in rats (Friedman & Tordoff 1987). In addition, it has been shown in rats on a medium fat (18%) diet, that although FFA levels are increased for as long as 6 hours after administration, the stimulation of food intake is only seen for 2 hours (Scharrer & Langhans 1986). MA also inhibits fatty acid re-esterification in adipose tissue and thus stimulates fatty acid mobilisation (Sabourault *et al* 1977). It has been suggested that the prolonged

elevation of FFA levels after MA administration may only be in part due to inhibition of fatty acid oxidation (Scharrer & Langhans 1986).

The observation that decreased oxidation of fatty acids induced by mercaptoacetate did not acutely increase food intake in *S. crassicaudata*, is similar to what occurs in deer mice (*Peromyscus maniculatus*) (Stamper & Dark 1997), but is in contrast to other mammals including rats and mice (Scharrer & Langhans 1986, DelPrete *et al* 1998). For example, in rats fed a medium fat (18%) diet, MA (400  $\mu\text{mol/kg}$  IP) increases food intake by as much as 120% for up to 6 hours following administration (Scharrer & Langhans 1986), whereas in adult deer mice housed at 15 °C, MA (1200  $\mu\text{mol/kg}$ ) fails to influence food intake (Stamper & Dark 1997). In addition, Syrian hamsters (which fail to increase food intake following a fast or after 2-DG administration) fail to respond to methyl palmoxirate induced inhibition of fatty acid oxidation by increasing food intake (Lazzarini *et al* 1988, Schneider *et al* 1988).

The increased food intake observed in *S. crassicaudata* with MA 200  $\mu\text{mol/kg}$  during the light phase was only at the 24-hour time point, and it is therefore difficult to conclude whether the increase in food intake was the result of inhibition of fatty acid oxidation, or was a non-specific effect. Similarly, the finding that MA 100  $\mu\text{mol/kg}$  decreased food intake under both light and dark conditions is difficult to explain. It is likely, however, that the highest dose (MA 800  $\mu\text{mol/kg}$ ) induced a decrease in food intake during the dark phase because it was making the animals feel unwell. High doses of MA (800  $\mu\text{mol/kg}$ ) have previously been shown to decrease food intake in rats (Langhans & Scharrer 1987), and high doses of methyl palmoxirate have been

shown to decrease food intake in Syrian hamsters (Lazzarini *et al* 1988). It therefore appears that, at least at high doses, these inhibitors of fatty acid oxidation have toxic effects.

It is unknown whether photoperiod has any effect on fatty acid oxidation in *S. crassicaudata*. Although there was little difference in the effect of MA on feeding between the light and dark phase, it appears that *S. crassicaudata* may be more sensitive to the effect of MA during the dark cycle because 800  $\mu\text{mol/kg}$  did not induce illness during the light phase. A possible explanation for the observed effect is the nocturnal nature of feeding in this species. *S. crassicaudata* consume the majority of food during the dark phase (see chapter 4) and therefore, at the onset of the dark phase, when the feeding drive is high, a dose of MA high enough to induce illness may have a greater effect on the animals' appetite than during the light phase, when the feeding drive is low.

The failure of MA to induce feeding in *S. crassicaudata* does not eliminate the possibility that FFA are utilised in the regulation of feeding in this species. It is unlikely that negative results obtained were due to a type 2 error, as the experiment has been repeated several times with comparable results. Numerous studies in rats have demonstrated that the fat content of the diet can affect the response to inhibitors of fatty acid oxidation (Singer *et al* 1997, 1998, Singer-Koegler *et al* 1996, Wang *et al* 1994). For example, much lower doses of MA stimulate feeding in rats fed high (66.4%) when compared to low (4.3%) fat diets (Singer-Koegler *et al* 1996), and MA increases food intake of a corn oil (unsaturated fat) but not a tallow (saturated fat)

diet in rats (Wang *et al* 1994). In the wild, *S. crassicaudata* exist on a diet of arthropods (Morton *et al* 1983) that are high in fat and protein. The standard laboratory diet is comparatively lower in fat (5% by wet weight). It is possible that MA may have effects on appetite in *S. crassicaudata* fed a more natural diet that is higher in fat content. It would therefore be of interest to repeat these experiments using mealworms (a diet more closely approximating the natural insectivorous diet) as a food source, rather than laboratory diet.

The reason for variation in the responses to fatty acid oxidation between species is unclear. Different inhibitors of fatty acid oxidation and different mechanisms of action of these inhibitors may be involved. Although it has been suggested that Syrian hamsters simply fail to respond to alterations in metabolic fuels due to differences in the nature of their satiety signals, this has not been confirmed (DiBattista & Bedard 1987, Lazzarini *et al* 1988). In rats, the increased feeding following inhibition of fatty acid oxidation is likely to be triggered by a signal from the liver, as both total and hepatic branch vagotomy attenuate or eliminate the effects of MA on food intake (Langhans *et al* 1985c, Ritter & Taylor 1990).

A decrease in fatty acid oxidation *per se* may not be sufficient to generate increased food intake (Friedman *et al* 1999). A limited capacity to oxidise alternative fuels, and a decrease in hepatic fatty acid oxidation, may stimulate feeding behaviour by limiting hepatic energy production (Friedman *et al* 1999). Evidence is accumulating that suggests changes in liver ATP, a product of oxidative phosphorylation, may be the stimulus that controls food intake (Friedman *et al* 1998, Friedman & Stricker 1976,

Rawson *et al* 1994). The liver has been demonstrated to play a major role in regulating energy homeostasis in mammals (VanDenBerghe 1991, Wasserman & Cherrington 1991). Interestingly, low levels of mitochondrial uncoupling protein-2 (UCP2) have been detected in macrophages, but not in hepatocytes of liver in lean rats (Larrouy *et al* 1997). In contrast, UCP2 is expressed in hepatocytes of genetically obese mice with fatty livers, suggesting that hepatocytes adapt to obesity by up-regulating UCP2 (Chavin *et al* 1999). Recent data has shown that circulating FFA concentration of rats correlates with UCP2 and UCP3 induction in white fat and skeletal muscle respectively, and that lipids up-regulate UCP2 expression in hepatocytes, suggesting that increased expression of UCPs may represent a metabolic adaptation of these tissues to increased fatty acid supply (Boss *et al* 1998). The liver may therefore adapt to an increased supply of lipid substrates by inducing UCP2 to facilitate substrate disposal.

*S. crassicaudata*, like the Syrian hamster, fails to respond to inhibition of fatty acid oxidation using mercaptoacetate. The role of fatty acid oxidation in the regulation of food intake in *S. crassicaudata* therefore remains unclear, but presumably there are mechanisms by which alterations in nutrient partitioning can be sensed. It would be of interest to examine the effect on food intake of other fatty acid inhibitors such as etomoxir or methyl palmoxirate, or the effect of nicotinic acid (which inhibits free fatty acid mobilisation from adipose tissue, facilitating a decrease in free fatty acid uptake by the liver and muscle) (Capurso 1991), to further clarify the role that free fatty acids play in appetite regulation of *S. crassicaudata*.

## CHAPTER 10

**PERIPHERAL ADMINISTRATION OF CORTICOTROPHIN  
RELEASING FACTOR OR UROCORTIN: EFFECTS ON FOOD  
INTAKE AND THE HPA AXIS IN *S. CRASSICAUDATA*****10.1 SUMMARY**

Peptides found within the hypothalamus, including corticotrophin releasing factor (CRF) and urocortin (UCN) decrease food intake and increase energy expenditure when administered both centrally and peripherally to rodents, however the effects of CRF and UCN on food intake in other mammals (for example marsupials) are not known. The aims of this study were to determine (i) the effects of peripheral administration of CRF and UCN on food intake, thermogenesis and plasma cortisol in the marsupial *S. crassicaudata*, and (ii) the receptor/s mediating these effects. *S. crassicaudata* were injected intraperitoneally (IP) with either saline, CRF (10–1000 µg/kg), or UCN (5–15 µg/kg), with and without the specific CRF1 receptor antagonist antalarmin (ANT) (20–50 mg/kg), injected IP 15 minutes beforehand. Energy intake, plasma cortisol, and metabolic parameters were measured in separate experiments.

UCN at 10 and 15 µg/kg suppressed energy intake by 23% ( $P \leq 0.05$ ) and 34% ( $P =$

0.01) respectively in the first 30 minutes, when compared to controls. CRF at 10 and 100  $\mu\text{g/kg}$  had no effect on energy intake, but in doses of 500 and 1000  $\mu\text{g/kg}$ , CRF decreased energy intake by 23% over the first 120 minutes ( $P \leq 0.05$ ), and by 30% over the first 60 minutes ( $P = 0.02$ ), respectively. ANT did not modify the effects of CRF or UCN on energy intake. There was no effect of CRF (1000  $\mu\text{g/kg}$ ) or UCN (15  $\mu\text{g/kg}$ ) on either oxygen consumption ( $\text{VO}_2$ ) or the respiratory quotient (RQ). After IP saline or CRF (0.5, 1, 10, 100, and 1000  $\mu\text{g/kg}$ ), plasma cortisol levels were 7.9, 9.8, 22.3, 26.9, 61.9 and 79.3 nmol/l respectively ( $P = 0.002$ ), whereas IP UCN (10-15  $\mu\text{g/kg}$ ) had no effect on plasma cortisol. ANT (20 mg/kg) abolished the response of plasma cortisol to doses of CRF up to and including 10  $\mu\text{g/kg}$ , but had no effect on the cortisol response to higher doses of CRF. Higher doses of ANT (50-100 mg/kg) produced seizures.

It is concluded that: (i) CRF induces cortisol release in *S. crassicaudata*, as in other mammals, via the CRF1 receptor, and (ii) when administered peripherally, UCN is ~50 fold more potent than CRF in reducing food intake. These observations indicate that while the CRF1 receptor predominantly regulates the HPA axis, the CRF2 receptor is primarily involved in the regulation of food intake, and that peripherally released UCN may be important in this regard.

## 10.2 INTRODUCTION

Glucocorticoids play a prominent role in the regulation of energy homeostasis (Dallman *et al* 1993). The regulation of cortisol production involves the hypothalamic peptide corticotrophin releasing factor (CRF), named for its ability to induce adrenocorticotrophin hormone (ACTH) release from corticotrophs of the anterior pituitary gland. CRF also has a number of other effects; when administered centrally in mammals (Drescher *et al* 1994, Glowa *et al* 1992, Parrot 1990, Rivest & Richard 1989), goldfish (DePedro *et al* 1997) and chicken (Denbow *et al* 1999), CRF decreases food intake. In addition, central administration of CRF has been shown to increase energy expenditure in rats, although this effect may be species specific (Drescher *et al* 1994, Emeric 1986).

Two subtypes of the CRF receptor (CRF1, CRF2) have been characterised, based on their pharmacological specificity and regional localisation in the rat, mouse, chicken and human (Chen *et al* 1993, Grigoriadis *et al* 1996, Perrin *et al* 1995, Yu *et al* 1996). The ACTH releasing affect of CRF is mediated by the CRF1 receptor (Smith *et al* 1998, Turnbull & Rivier 1997) that, in rodents, is expressed in the pituitary and brain (Chang *et al* 1993, Perrin *et al* 1993, Vita *et al* 1993). The CRF2 receptor is expressed in brain and in peripheral tissues, predominantly in the gastrointestinal tract and heart (Kishimoto *et al* 1995, Lovenberg *et al* 1995, Perrin *et al* 1995). The endogenous ligand for the CRF2 receptor is now known to be another member of the 'CRF family', the 40 amino acid peptide urocortin (UCN) (Vaughan *et al* 1995). In rats, UCN shows 63% homology to urotensin and 45% homology to CRF. *In vitro*



studies have demonstrated that although UCN binds to both CRF1 and 2 receptors with greater affinity than CRF, its affinity for the CRF2 receptor is 40 times greater than that of CRF, whereas the affinity for the CRF1 receptor is only 3-10 times greater (Vaughan *et al* 1995).

When administered centrally to rats, UCN is more potent than CRF in decreasing food intake (Spina *et al* 1996); the anorexic effects of both CRF and UCN appear to be mediated (at least centrally) by the CRF2 receptor (Smagin *et al* 1998). Both UCN and the CRF2 receptor are abundant in the gastrointestinal tract, suggesting a paracrine role for UCN on gut function (Lovenberg *et al* 1995, Perrin *et al* 1995, Vaughan *et al* 1995). In rodents, peripheral administration of both UCN and CRF has recently been shown to slow gastric emptying and decrease food intake, but, as with central administration, UCN is more potent than CRF in this regard, and these effects appear to be mediated by the CRF2 receptor (Asakawa *et al* 1999, Nozu *et al* 1999).

A number of CRF receptor antagonists have been developed and used to clarify the role of CRF1 and 2 receptors in the biological actions of UCN and CRF. These include anti-sauvagine-30, a CRF2 receptor antagonist, and NBI-27914, which antagonise CRF1 receptor mediated effects (Chen C *et al* 1996, Ruhmann *et al* 1998). Antalarmin (ANT), a synthetic pyrrolo-pyrimidine compound, antagonises CRF1 receptor-mediated effects, blocking pituitary ACTH release, stress behaviours, and acute inflammation (Schultz *et al* 1996, Webster *et al* 1996). The acute effect of CRF1 receptor antagonism using ANT on food intake has not been determined.

Although chronic peripheral administration of ANT has no effect on body weight in rats (Bornstein *et al* 1998), its effects on energy intake are unknown.

The amino acid sequence of CRF demonstrates a high level of homology among species, suggesting conservation of physiological function (Gillies *et al* 1989, Owens & Nemeroff 1991). Despite evidence for species-specific effects on energy expenditure (Drescher *et al* 1994, Emeric 1986), the central effect of CRF on appetite across a range of vertebrate species appears to be highly conserved (Denbow *et al* 1999, DePedro *et al* 1997, Drescher *et al* 1994, Glowa *et al* 1992, Rivest & Richard 1989). To date however, peripheral effects of CRF and UCN on appetite have only been demonstrated in rodents; there are no studies that have examined the effects of peripherally administered CRF or UCN on appetite or energy expenditure in other vertebrate species, such as marsupials.

The aims of this study were to determine the effects of peripheral CRF and UCN administration on food intake, energy expenditure and plasma cortisol in the marsupial *S. crassicaudata*, and the receptors involved in mediating these effects.

## 10.3 MATERIALS AND METHODS

### 10.3.1 *Experimental animals, diet and drugs*

Adult male *S. crassicaudata* housed on a long day photoperiod (16-h light, 8-h dark; lights off 15:00h), were used. Animals were fed standard laboratory diet *ad libitum* except where stated. Food intake is expressed as kilocalories (kcal).

Urocortin (UCN), corticotrophin releasing factor (CRF), and antalarmin (ANT) were prepared as outlined in section 3.5.6. All drugs were administered intraperitoneally (IP).

### 10.3.2 *Metabolic variables, blood sampling, plasma cortisol measurement*

Whole-body oxygen consumption ( $\text{VO}_2$ ) and respiratory quotient (RQ) were determined using previously established methods (see section 3.3.4). Baseline  $\text{VO}_2$  was measured over a 2-hour period to allow animals to reach a 'steady state'. Animals were then injected IP with either saline, UCN (15  $\mu\text{g}/\text{kg}$ ), or CRF (1000  $\mu\text{g}/\text{kg}$ ) and returned to the metabolic chamber.  $\text{VO}_2$  was measured every 15 minutes for a further 2 hours.

Blood was obtained as described in section 3.4, spun at 4 °C for 15 minutes and the plasma immediately assayed for cortisol using the method outlined in section 3.4.3.

### 10.3.3 *Experimental protocols*

#### Study 1: Effects of UCN, CRF and ANT on energy intake

The effects of UCN, CRF, and ANT on deprivation-induced feeding were determined in groups of *S. crassicaudata* ( $n = 10-17$ ), matched for body weight. Following a period of 24-hour food deprivation, animals were weighed, and injected IP at lights out (15:00h), with either vehicle, UCN (5, 10, or 15  $\mu\text{g/kg}$ ), CRF (10, 100, 500, 1000  $\mu\text{g/kg}$ ) or ANT (20, 50, or 100  $\text{mg/kg}$ ). Pre-weighed laboratory diet was introduced into the cage immediately following UCN and CRF administration, and 15 minutes after the administration of ANT. Animals were then allowed to eat *ad libitum*, and subsequent food intake recorded at 0.5, 1, 2, 3, and 24 hours. Different doses of each drug and vehicle control were given in a counter-balanced order in tests 7 days apart, such that no animal received more than one dose of drug within a 7 day period.

#### Study 2: Effects of ANT on feeding following UCN or CRF administration

To determine whether the specific CRF1 receptor antagonist ANT (20 or 50  $\text{mg/kg}$ ) influences the effect of UCN (10, 15  $\mu\text{g/kg}$ ) and CRF (500, 1000  $\mu\text{g/kg}$ ) on deprivation induced feeding, the study was carried out as in study 1 ( $n = 10-15$  per group). However, 15 minutes prior to injection with either saline, UCN or CRF, animals were injected IP with either the ANT 'control' solution, or ANT (20 or 50  $\text{mg/kg}$ ).

Study 3: Effects of UCN and CRF on metabolic variables

The effects of UCN (15 µg/kg) and CRF (1000 µg/kg) on VO<sub>2</sub> and RQ were determined using 6 male *S. crassicaudata*. 'Steady state' VO<sub>2</sub> was obtained for each animal, and animals were then injected IP with either saline, UCN or CRF. Subsequent VO<sub>2</sub> and RQ were determined every 15 minutes for 2 hours following injection. Animals received each treatment in a randomised manner, with at least 7 days between each treatment, such that each animal acted as its own control.

Study 4: Effects of UCN and CRF on plasma cortisol levels, and whether any effect is modified by ANT

To determine the effects of UCN and CRF on plasma cortisol, *S. crassicaudata* ( $n = 4-20$  per group) were injected IP with either saline, UCN (10, 15 µg/kg) or CRF (0.5, 1, 10, 100, 500, 1000 µg/kg). Blood was sampled 40 minutes following injection and plasma assayed for cortisol.

To determine if ANT modified the cortisol response to UCN or CRF, *S. crassicaudata* ( $n = 6-16$  per group) were injected IP with either saline or ANT (20 mg/kg), 15 minutes prior to IP CRF (10, 100, 1000 µg/kg) administration. Blood was sampled 40 minutes later and assayed for plasma cortisol.

**10.3.4 Statistical analyses**

Statistical analyses were performed as outlined in section 3.9.

## 10.4 RESULTS

### 10.4.1 Study 1: Effects of UCN, CRF and ANT on energy intake

Baseline body weights did not differ significantly between the groups.

*UCN*: UCN, 5 µg/kg, had no effect on energy intake at any time point, whereas UCN, 10 µg/kg, decreased energy intake by 23% in the first hour only ( $F_{1,47} = 3.96$ ,  $P = 0.05$ ), Fig 10.1. UCN, 15 µg/kg, induced a 34% decrease in energy intake in the first hour ( $F_{1,67} = 6.21$ ,  $P = 0.01$ ), and over 3 hours a 16% decrease in energy intake occurred ( $F_{1,101} = 5.07$ ,  $P = 0.03$ ), Fig 10.1.

*CRF*: CRF, 10 and 100 µg/kg, had no effect on energy intake at any time point. CRF, 500 µg/kg, decreased energy intake by 12% in the first hour ( $F_{1,43} = 3.28$ ,  $P = 0.09$ ) and by 2 hours, overall energy intake was reduced by 23% ( $F_{1,65} = 4.14$ ,  $P = 0.05$ ), Fig 10.2. CRF, 1000 µg/kg, decreased energy intake by 30% in the first hour ( $F_{1,43} = 6.65$ ,  $P = 0.02$ ), and over 3 hours a 24% reduction in energy intake occurred ( $F_{1,87} = 5.89$ ,  $P = 0.02$ ), Fig 10.2.

*ANT*: ANT, 20 mg/kg alone, had no effect on energy intake at any time point. ANT at 50 mg/kg ( $F_{1,109} = 22.3$ ,  $P < 0.0001$ ), and 100 mg/kg ( $F_{1,114} = 10.8$ ,  $P = 0.004$ ), induced a decrease in energy intake at all time-points, Fig 10.3. Latter doses (50, 100 mg/kg) induced seizures in many animals, which appeared to last for up to 2-4 hours.

#### 10.4.2 Study 2: Effects of ANT on feeding following UCN or CRF administration

Baseline body weights did not differ significantly between the groups.

The effects of UCN at 10 and 15  $\mu\text{g/kg}$ , and CRF at 500 and 1000  $\mu\text{g/kg}$  on energy intake, were not attenuated by prior administration of ANT 20  $\text{mg/kg}$ . The prior administration of ANT 50  $\text{mg/kg}$  did not attenuate the decreases in energy intake that occurred in response to UCN 10  $\mu\text{g/kg}$  or CRF 500  $\mu\text{g/kg}$ , Figs 10.4 a,b.

While ANT, 20  $\text{mg/kg}$  alone, had no effect on food intake, the combinations of ANT 20  $\text{mg/kg}$  + UCN 10  $\mu\text{g/kg}$ , and ANT 20  $\text{mg/kg}$  + UCN 15  $\mu\text{g/kg}$  induced a greater decrease in energy intake in the first hour (44%,  $F_{1,59} = 6.14$ ,  $P = 0.02$ , and 39%,  $F_{1,47} = 6.61$ ,  $P = 0.02$  respectively) when compared to UCN (10, 15  $\mu\text{g/kg}$ ) alone. Over 3 hours, a reduction in energy intake was also observed in both groups (45%,  $F_{1,119} = 7.39$ ,  $P = 0.01$ , and 26%,  $F_{1,95} = 7.45$ ,  $P = 0.01$ ), Fig 10.4a. Similarly, the combination of ANT 20  $\text{mg/kg}$  + CRF 500  $\mu\text{g/kg}$ , and ANT 20  $\text{mg/kg}$  + CRF 1000  $\mu\text{g/kg}$  induced a greater decrease in energy intake in the first hour (46%,  $F_{1,41} = 4.40$ ,  $P = 0.04$ , and 39%,  $F_{1,43} = 13.15$ ,  $P = 0.002$  respectively) when compared to CRF (500, 1000  $\mu\text{g/kg}$ ) alone. Over 3 hours a reduction in energy intake was also observed in both groups (44%,  $F_{1,83} = 5.94$ ,  $P = 0.02$ , and 26%,  $F_{1,95} = 7.45$ ,  $P = 0.01$ ), Fig 10.4b.

The combinations ANT 50  $\text{mg/kg}$  + UCN 10  $\mu\text{g/kg}$ , and ANT 50  $\text{mg/kg}$  + CRF 500  $\mu\text{g/kg}$  induced greater decreases in energy intake in the first hour (78%,  $F_{1,43} = 16.78$ ,

$P = 0.0006$ , and 77%,  $F_{1,45} = 13.53$ ,  $P = 0.001$  respectively) when compared to UCN 10  $\mu\text{g/kg}$  or CRF 500  $\mu\text{g/kg}$  when given alone, Figs 10.4a, b.

#### 10.4.3 Study 3: Effects of CRF and UCN on $\text{VO}_2$ and RQ.

In the saline and UCN 15  $\mu\text{g/kg}$  groups, 'steady-state'  $\text{VO}_2$  was  $4.74 \pm 0.45$  and  $5.18 \pm 0.47$  ml/g/h, and RQ was  $0.66 \pm 0.02$  and  $0.68 \pm 0.02$  respectively, and there was no difference between them. UCN had no effect on  $\text{VO}_2$  or RQ over the 2 hours following injection, Fig 10.5a.

In the saline and CRF 1000  $\mu\text{g/kg}$  groups, "steady-state"  $\text{VO}_2$  was  $5.57 \pm 0.45$  and  $4.79 \pm 0.35$  ml/g/h, and RQ was  $0.54 \pm 0.004$  and  $0.51 \pm 0.02$  respectively, and there was no difference between them. CRF had no effect on  $\text{VO}_2$  or RQ over the 2 hours following injection, Fig. 10.5b.

#### 10.4.4 Study 4: Effects of UCN and CRF on plasma cortisol

UCN, 10 and 15  $\mu\text{g/kg}$ , had no effect on plasma cortisol levels as compared to saline treated controls, Fig 10.6a. CRF, 0.5  $\mu\text{g/kg}$ , had no effect on plasma cortisol levels, whereas CRF, 1  $\mu\text{g/kg}$  ( $P = 0.04$ ), 10  $\mu\text{g/kg}$  ( $P = 0.01$ ), 100  $\mu\text{g/kg}$  ( $P = 0.002$ ), 500  $\mu\text{g/kg}$  ( $P = 0.0001$ ) and 1000  $\mu\text{g/kg}$  ( $P = 0.001$ ) induced a dose-dependent increase in plasma cortisol compared to saline treated controls ( $F_{8,76} = 9.20$ ,  $P < 0.0001$ ), Fig 10.6a.

Animals injected twice with saline ( $P = 0.001$ ), or once with the ANT 'control' and once with saline ( $P = 0.02$ ), had increased cortisol levels compared to those animals



given only one injection of saline. ANT 20 mg/kg, injected prior to saline, prevented the increased cortisol response observed in response to two saline injections, Fig 10.6b.

In animals pre-injected with saline and then CRF at 10 or 100  $\mu\text{g/kg}$ , plasma cortisol was higher when compared to the saline + saline injected ( $P = 0.05$ ,  $P = 0.0007$  respectively) and the 'control' + saline injected ( $P = 0.05$ ,  $P < 0.0001$  respectively) groups. Pre-treatment with ANT (20 mg/kg) prevented the increase in plasma cortisol observed with CRF at 10  $\mu\text{g/kg}$  but did not ameliorate the effects of CRF 100 or 1000  $\mu\text{g/kg}$ , to increase plasma cortisol, Fig 10.6b.

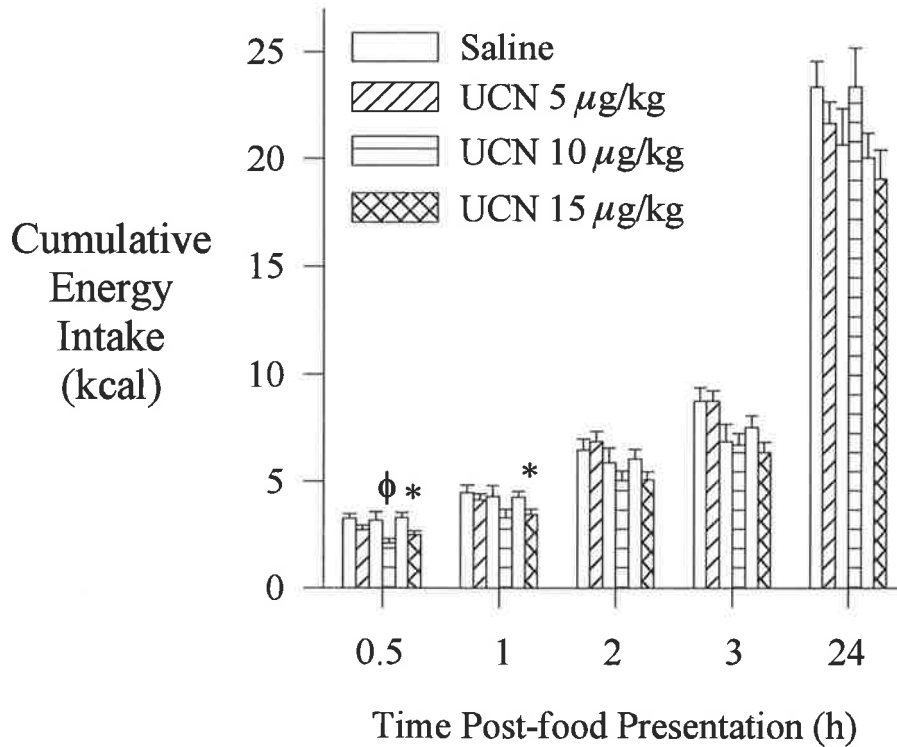


**Figure 10.1:** The effect of IP UCN (5, 10, 15 µg/kg) on deprivation-induced feeding in male *S. crassicaudata*, compared to saline treated controls, ( $n = 12-17$ ). UCN 10 µg/kg, reduced energy intake for 1 hour ( $P = 0.05$ ), and UCN 15 µg/kg, reduced energy intake for up to 3 hours ( $P = 0.03$ ). Values are Mean  $\pm$  SEM; \*  $P < 0.05$ , ϕ  $P < 0.01$ , compared to saline control.

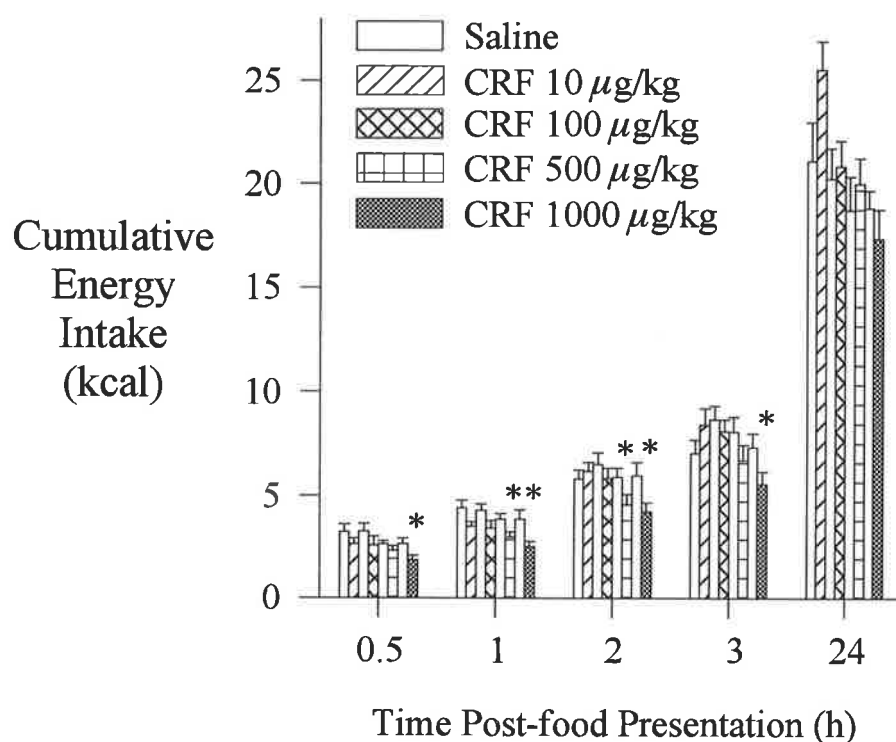


**Figure 10.2:** The effect of IP CRF (10, 100, 500, 1000  $\mu\text{g/kg}$ ) on deprivation-induced feeding in male *S. crassicaudata*, compared to saline treated controls, ( $n = 11-12$ ). CRF 500  $\mu\text{g/kg}$ , reduced energy intake for 2 hours ( $P = 0.05$ ), and CRF 1000  $\mu\text{g/kg}$ , decreased energy intake for up to 3 hours ( $P = 0.02$ ). Values are mean  $\pm$  SEM; \*  $P < 0.05$ , compared to saline control.

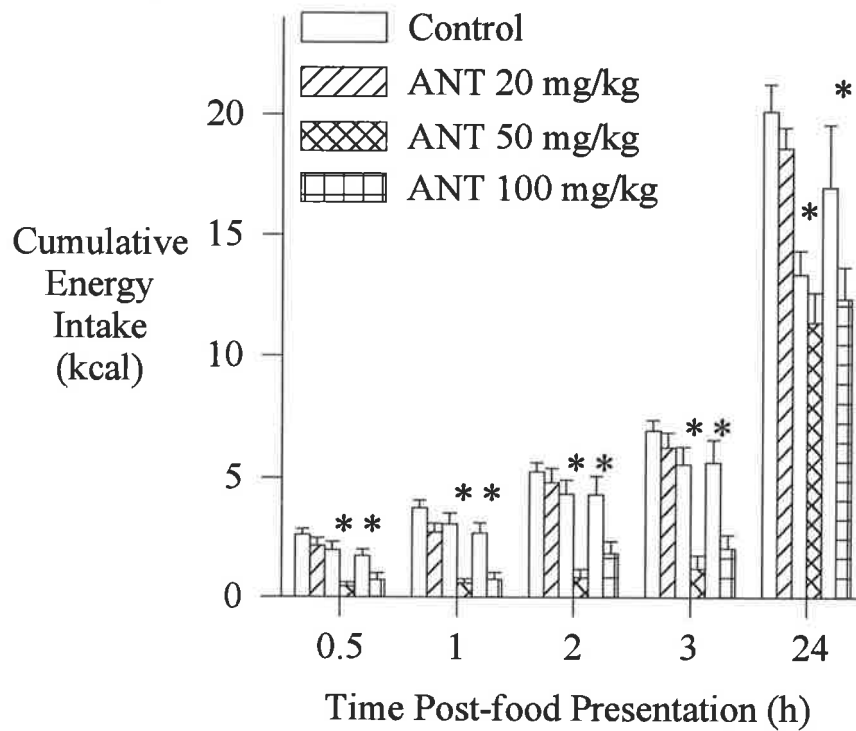


**Figure 10.3:** The effect of IP ANT (20, 50, 100 mg/kg) on deprivation-induced feeding in male *S. crassicaudata* compared to 'control' treated animals, ( $n = 10-12$ ). ANT 20 mg/kg, had no effect on energy intake, whereas ANT 50 mg/kg ( $P < 0.0001$ ), and 100 mg/kg ( $P = 0.004$ ) decreased energy intake for up to 24 hours. Values are mean  $\pm$  SEM; \*  $P < 0.05$ , compared to 'control'.

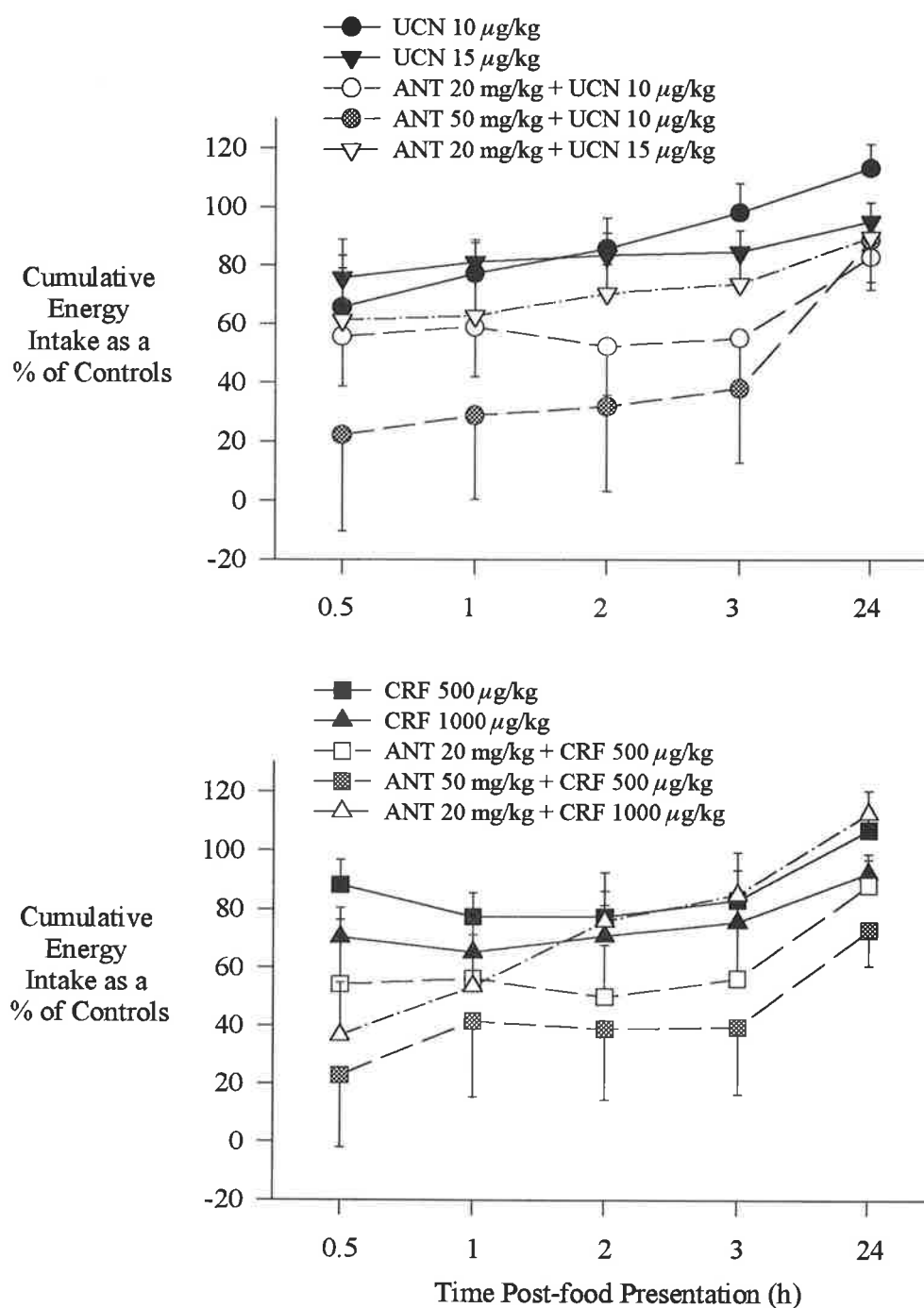


**Figure 10.4:** Effects of IP ANT (20, 50  $\text{mg/kg}$ ) pre-treatment on cumulative energy intake as a % of control intake, in response to IP UCN (10, 15  $\mu\text{g/kg}$ ) (top panel), and CRF (500, 1000  $\mu\text{g/kg}$ ) (bottom panel), ( $n = 10-15$ ). ANT did not modify the response to either UCN or CRF. Mean  $\pm$  SEM.

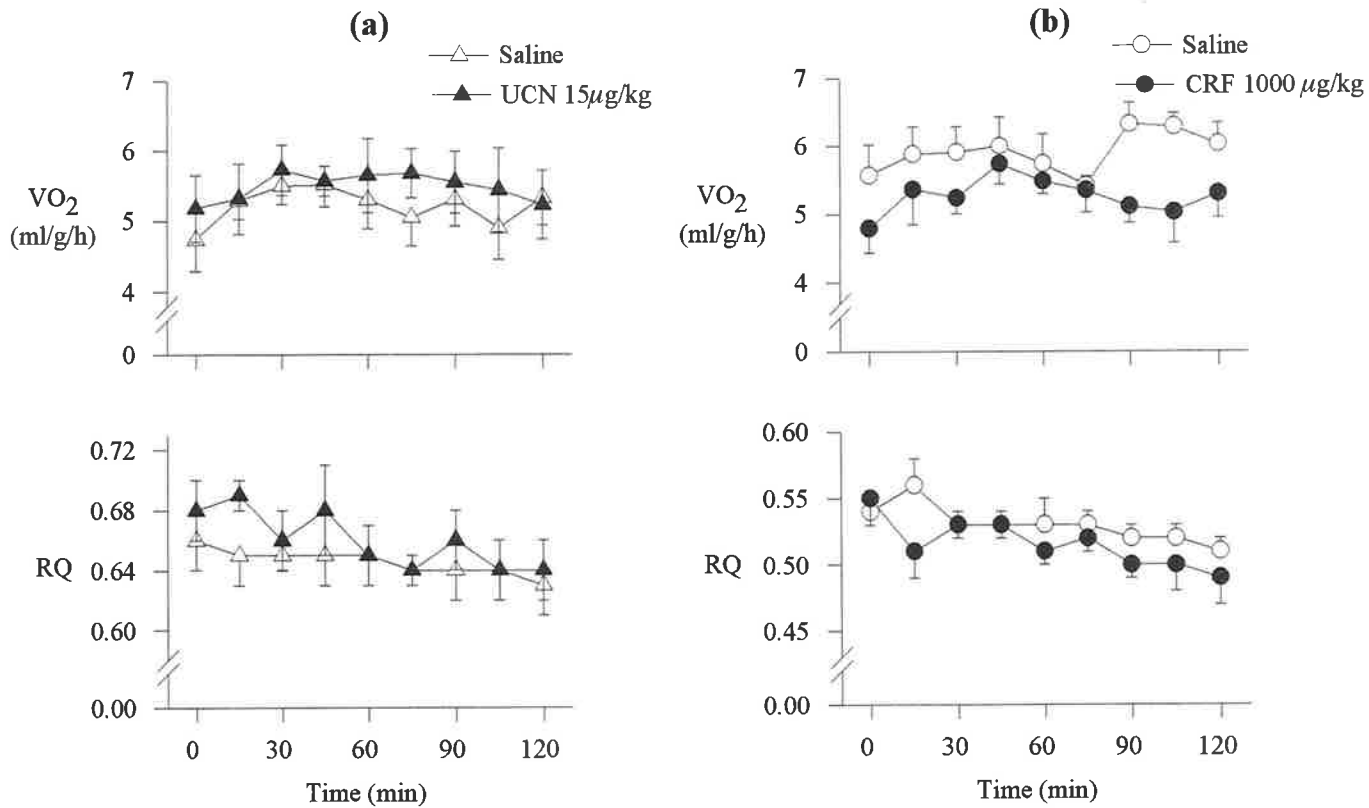


**Figure 10.5:** Effects of (a) UCN 15  $\mu\text{g/kg}$ , and (b) CRF 1000  $\mu\text{g/kg}$ , on  $VO_2$  and RQ in male *S. crassicaudata*, compared to saline treated controls, ( $n = 6$ ). UCN and CRF were administered IP once animals had reached 'steady-state'; measurements were taken every 15 minutes for the subsequent 2 hours. Neither UCN nor CRF had any effect on  $VO_2$  or RQ. Mean  $\pm$  SEM.

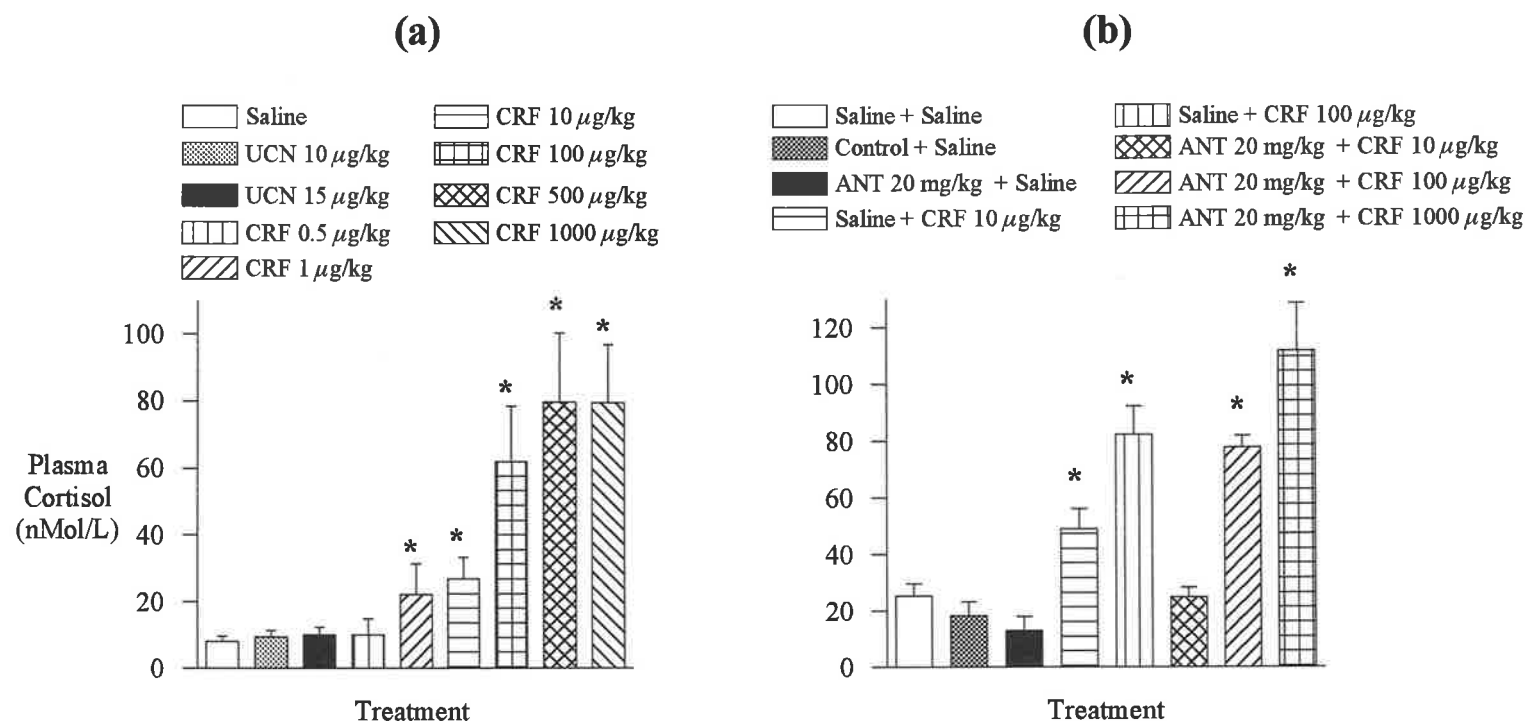


**Figure 10.6:** Effects of IP (a) UCN (10, 15  $\mu$ g/kg), and CRF (0.5, 1, 10, 100, 500, 1000  $\mu$ g/kg), ( $n = 4-20$ ), and (b) ANT (20 mg/kg) treatment 15 minutes before CRH administration ( $n = 6-16$ ), on plasma cortisol, 40 minutes after treatment. UCN had no effect on plasma cortisol, nor did CRF, 0.5  $\mu$ g/kg. CRF concentrations above 0.5  $\mu$ g/kg increased plasma cortisol in a dose-dependent manner ( $P < 0.0001$ ). Administration of ANT (20 mg/kg) attenuated the effect of CRF 10  $\mu$ g/kg to increase plasma cortisol, but had no effect on the increase in plasma cortisol observed with CRF 100, 1000  $\mu$ g/kg. Values are mean  $\pm$  SEM; \*  $P < 0.05$  as compared to saline control.

## 10.5 DISCUSSION

This study has demonstrated that peripherally administered urocortin (UCN) and corticotrophin-releasing factor (CRF) induce short-term decreases in energy intake of fasted *S. crassicaudata*. The dose of CRF required to decrease energy intake is approximately 50 times that of UCN, implying that peripheral UCN is a more potent anorectic factor. The doses of CRF and UCN that decreased energy intake had no effect on whole body oxygen consumption ( $\text{VO}_2$ ) or respiratory quotient (RQ). Peripheral UCN (in the doses tested) did not effect plasma cortisol levels, whereas CRF increased plasma cortisol levels in a dose-dependent manner. The CRF1 receptor antagonist antalarmin (ANT) prevented the increase in cortisol levels seen with lower doses of CRF, but did not attenuate the effects of UCN and CRF on energy intake, suggesting that the peripheral effects of UCN and CRF are mediated primarily via the CRF2 receptor.

There is little information about the regulation of the hypothalamic pituitary axis (HPA) in marsupials. Peripherally administered CRF induces a dose-dependent, CRF1 receptor mediated increase in plasma cortisol in *S. crassicaudata*, as in other mammals. The specific CRF1 receptor antagonist ANT abolished not only the effect of CRF at lower doses, but also prevented the increase in cortisol levels observed in response to the stress of two consecutive injections, suggesting that the response to this stress is also mediated via CRF acting at the CRF1 receptor. This is consistent with the pivotal role that the CRF1 receptor plays in regulating the response of the HPA axis to stress in other mammals. The response of the HPA axis to stress is



impaired in the CRF1 receptor knockout mouse (Smith *et al* 1998). CRF1 receptor antagonists (ANT, NBI-27914, SC-241) have been shown to abolish the effect of centrally administered CRF to stimulate ACTH release in other animals (Chen C *et al* 1996, Chen *et al* 1997, Webster *et al* 1996).

UCN, at the doses tested, failed to stimulate cortisol secretion in *S. crassicaudata*, even though equivalent doses of CRF did. It is possible that higher doses of UCN may have elicited a response, however these observations are consistent with those of Asakawa *et al* (1999) showing no effect of peripheral UCN on corticosterone levels when administered chronically (3 nmol/mouse for 5 days) to *ob/ob* mice (Asakawa *et al* 1999). *In vitro* UCN is 8 times more potent than CRF at stimulating ACTH release from anterior pituitary cells of rats (Vaughan *et al* 1995), and UCN is more potent than CRF at increasing plasma cortisol and ACTH when given intravenously (i.v.) to sheep (Parkes *et al* 1997), suggesting species-specific differences.

When administered centrally, both UCN and CRF decrease food intake in all species so far studied, with UCN being consistently more potent than CRF in this regard (Smagin *et al* 1998, Spina *et al* 1996, Vaughan *et al* 1995). While central administration of CRF (1 µg) decreases food intake in both rats (Negri *et al* 1985) and goldfish (*Carassius auratus*) (DePedro *et al* 1997), peripheral administration of (0.2 µg/g in rats, and 0.1 µg/g in goldfish), has no effect on food intake. As in other animals, recent studies have found that CRF (1 µg) decreases energy intake when administered centrally to *S. crassicaudata* (Morley JE, personal communication). In the current study, peripherally administered CRF decreased energy intake only at

doses of 500 and 1000  $\mu\text{g/kg}$ . In contrast, relatively low doses of UCN (10,15  $\mu\text{g/kg}$ ) administered peripherally decrease food intake in *S. crassicaudata*, as in mice (Asakawa *et al* 1999).

The selective CRF1 receptor antagonist ANT, in doses that inhibited the response of the HPA axis to CRF, failed to prevent the decrease in energy intake observed in response to peripherally administered UCN or CRF in *S. crassicaudata*. these observations suggest that the effects of peripheral UCN and CRF on energy intake are mediated primarily via the CRF2 receptor. While ANT (20 mg/kg) had no effect on food intake, higher doses decreased food intake, and when combined with CRF or UCN, the reduction in food intake was greater than with either peptide alone. High doses of ANT also resulted in seizures. Both of these effects therefore limit the potential usefulness of ANT as a CRF1 antagonist. Nevertheless, the dose of ANT that was not associated with toxicity was sufficient to attenuate the effect of CRF on induce cortisol release, suggesting that, at least within the physiological range, CRF1 receptor antagonism was adequate and the timing of administration appropriate.

Based on the studies of others, it is unlikely that the CRF1 receptor plays a significant role in either the central or peripheral effects of CRF or UCN on energy intake (Asakawa *et al* 1999, Bornstein *et al* 1998, DePedro *et al* 1997, Smagin *et al* 1998). For example, in rats, administration of antisense oligonucleotides to CRF2 receptor mRNAs significantly attenuates CRF and UCN induced decreases in food intake (Smagin *et al* 1998), suggesting that this effect is mediated primarily by the CRF2 receptor, or at least one of its sub-types. The concept that the peripheral actions of

UCN are mediated by the CRF2 receptor is supported by circumstantial evidence only, and this issue is not fully settled in any species. The CRF1 and CRF2 receptors have not been cloned from *S. crassicaudata* and it is therefore not possible to determine the relative affinity of the different peptide ligands for these receptors. However, since the response of plasma cortisol to CRF, UCN and ANT in *S. crassicaudata* is similar to that observed in rodents, reasonable inferences regarding receptor selectivity may be made.

CRF2 receptors located within the ventromedial and paraventricular nuclei of the hypothalamus are responsible for the effects of centrally administered CRF and UCN on food intake and energy metabolism (Smagin *et al* 1998). Within the brain, CRF2 appears to be located within areas implicated in control of food consumption and digestive functions; for example, the paraventricular nucleus of the hypothalamus and dorsal vagal complex of the brain stem are both target sites of action of CRF-induced inhibition of gastric motor function. It is, however, unlikely that peripheral UCN or CRF inhibit food intake via central actions because of their limited ability to cross the blood brain barrier (Banks & Kastin 1996, Martins *et al* 1997), and a peripheral site of action therefore seems more likely. While it is possible that ANT has independent central effects on food intake, there is no evidence that this occurs when administered at a dose that inhibits peripheral CRF1 receptors.

Although not measured in this study, gastric emptying has been demonstrated to play a role in the regulation of food intake (Andrews *et al* 1998, Imeryuz *et al* 1997, Moran & McHugh 1982), and both central and peripheral CRF administration have

been demonstrated to decrease gastric emptying (Asakawa *et al* 1999, Nozu *et al* 1999, Sheldon *et al* 1990). Interestingly, other peptides known to be involved in appetite regulation, such as cholecystokinin and glucagon-like peptide 1 (Imeryuz *et al* 1997, Moran & McHugh 1982), also affect gastrointestinal motility. UCN administered IP (0.003-3 nmol) slows gastric emptying in both lean and *ob/ob* mice (Asakawa *et al* 1999), and i.v. UCN (1.2 - 4 µg/kg) decreases gastric emptying in rats (Nozu *et al* 1999). Peripheral UCN has been demonstrated to have a more potent effect than CRF in reducing gastric emptying (Asakawa *et al* 1999, Nozu *et al* 1999), and the CRF1 receptor antagonists ANT and NBI-27914 have no effect on i.v. CRF- or UCN-induced gastric stasis in rats (Chen C *et al* 1996, Nozu *et al* 1999). Taken together, these findings suggest that slowing of gastric emptying following peripheral CRF and UCN is primarily mediated by the CRF2 receptor. The slowing of gastric emptying or some other peripheral effect may therefore underlie the observed effects of UCN and CRF on food intake. In addition to its effects on gastric emptying, UCN decreases blood pressure (Torpy *et al* 1999), which may potentially contribute to reductions in energy intake. The effects of peripheral UCN and CRF on gastric emptying and blood pressure in *S. crassicaudata* remain to be determined.

It is possible that peripherally administered CRF and UCN induce illness in *S. crassicaudata* and thereby effect energy intake. Studies performed in this laboratory have demonstrated that in unwell animals  $VO_2$  is reduced (Clements *et al* 1998); this was not the case with the doses of CRF or UCN tested. No study has to date examined the effect of peripheral CRF, or either central or peripheral UCN, on  $VO_2$  or RQ. The failure of peripheral CRF to influence  $VO_2$  or RQ in *S. crassicaudata* is

consistent with the notion that CRF exerts its effects on energy expenditure centrally. The failure for UCN to affect  $\text{VO}_2$  is consistent with its suggested peripheral effects. In addition to effects on  $\text{VO}_2$ , centrally administered CRF has influences energy expenditure as a result of activation of brown fat thermogenesis (Brown *et al* 1982, Egawa *et al* 1990, LeFeuvre *et al* 1987).

The observation that CRF induces cortisol release in *S. crassicaudata* via the CRF1 receptor is consistent with studies in other mammals. Furthermore, the observation that UCN does not effect plasma cortisol when administered peripherally, but is  $\sim 50$  times more potent than CRF in suppressing food intake, is consistent with its role as a CRF2 receptor antagonist. Further studies using antagonists specific for the CRF2 receptor, such as antisauvagine, are required to further evaluate the hypothesis that the CRF2 receptor is responsible for effects of peripheral UCN and CRF on appetite.

## CHAPTER 11

**FOOD INTAKE AND FOOD CHOICE IN *S. CRASSICAUDATA*:  
THE ROLE OF THE ENDOGENOUS OPIOID PEPTIDES****11.1 SUMMARY**

Endogenous opioid peptides activate food seeking behaviour and influence macronutrient choice in a number of animal species. Previous studies in rodents and humans suggest that the palatability of food is also modulated by the opioid feeding system. The effect of opioid peptides on appetite and food choice in marsupials has not been evaluated. The aim of these studies was to determine the effect of  $\mu$ ,  $\delta$  and  $\kappa$  opioid receptors on food intake and food choice in the marsupial *S. crassicaudata*.

When offered a choice of mealworms or standard laboratory diet after 24-hour food deprivation, *S. crassicaudata* ate predominantly mealworms ( $P < 0.0001$ ). After a 24-hour fast, adult male *S. crassicaudata* were injected peripherally with opioid receptor antagonists specific for  $\mu$ ,  $\delta$ , or  $\kappa$  opioid receptors, or saline. Animals were re-fed with either laboratory diet alone, or a choice of laboratory diet and mealworms. In animals re-fed with laboratory diet alone, naloxone (predominantly a  $\mu$  receptor antagonist) at doses of 15 and 10 mg/kg reduced food intake by 31% and 38% respectively in the first 30 minutes after laboratory diet was re-introduced ( $P < 0.05$ ), but lower doses had no

effect. The selective  $\delta$  antagonist naltrindole in a dose of 20 mg/kg decreased food intake by 65% between 30-60 minutes ( $P < 0.01$ ). The selective  $\kappa$  opioid antagonist nor-binaltorphimine had no effect on the intake of laboratory diet. In animals offered a choice of laboratory diet and mealworms, naloxone in doses of 1, 5, 10, 15 and 20 mg/kg significantly decreased intake between 0-30 minutes, due to a preferential suppression of the intake of mealworms. Neither naltrindole nor nor-binaltorphimine had an effect on food choice. These studies establish that endogenous opioid peptides influence both food intake and choice in *S. crassicaudata*, and that the role of the opioid feeding system is in part modulated by food palatability. In *S. crassicaudata* these effects appear to occur predominantly by a  $\mu$  opioid receptor mechanism.

## 11.2 INTRODUCTION

Food choice is a complex process which is linked to reward behaviour. Although a number of peptides have been shown to regulate food intake, there is much less information about the factors that control food choice. The endogenous opioid peptides have been implicated in the reward or affective aspects of feeding, and have been shown to influence food choice, as well as food intake. Opioid agonists increase food intake in a wide variety of species including molluscs, domestic fowl, rats and mice, and humans (Denbow & McCormack 1990, DeZwaan & Mitchell 1992, Dum *et al* 1983, Gosnell *et al* 1986, Kavaliers & Hirst 1986, Levine *et al* 1990, Mann *et al* 1988, Morley *et al* 1982a, Yim & Lowry 1984). Opioids and feeding are discussed further in section 1.6.4.4. In rats, the anorectic potency of opioid antagonists, such as the  $\mu$ -preferring naloxone, is

greater when they are fed sweet foods rather than their usual laboratory diets (Cooper *et al* 1985a,b, Jackson & Cooper 1985, Levine *et al* 1982b, 1995). In humans, the  $\mu$ -preferring opioid antagonist naltrexone alters taste perception and decreases preference for sucrose (Bertino *et al* 1991, Fantino *et al* 1986). In some species, including rats and humans, endogenous opioid peptides have also been shown to influence macronutrient choice. For example, Yeomans *et al* (1990), found that the reduction in food intake in humans induced by the universal opioid antagonist nalmefene is associated with decreases in fat and protein intake, but not intake of carbohydrate. There is also evidence that opioid receptors are involved in the modulation of food palatability, as opioid receptor deficient mice exhibit a reduced preference for saccharin compared to control mice (Yirmiya *et al* 1988).

Although the effects of opioid peptides on feeding and food choice are thought to be mediated predominantly by  $\mu$  and  $\kappa$  opioid receptors (Morley 1995), only a few studies have compared intake of highly palatable foods to that of a regular laboratory diet in a single experiment; moreover, no studies have evaluated the effects of antagonists specific for each receptor type (*ie.*  $\mu$ ,  $\delta$ ,  $\kappa$ ) on both total food intake and food choice in a single study.

Not all species exhibit an opioid feeding system. For example, in the golden hamster (*Mesocricetus auratus*) drinking, but not feeding, is opiate sensitive (Lowry & Yim 1982), and in the raccoon (*Procyon lotor*) opioids do not appear to play a direct role in the regulation of food intake (Nizielski *et al* 1985). In the Chinese hamster (*Cricetulus griseus*), feeding is not affected by naloxone or butorphanol (a universal opioid



antagonist), nor does this species exhibit classical  $\mu$  or  $\kappa$  opioid binding sites in the brain (Billington *et al* 1984).

The observation that endogenous opioid peptides influence food intake in a wide range of animal groups supports the concept of an early evolution of opioid regulation of feeding. However, the role of endogenous opioid peptides in appetite regulation and food choice in marsupials is unknown. This study demonstrates that *S. crassicaudata* prefer mealworms to standard laboratory diet, and has subsequently examined the effect of opioid antagonists, selective for  $\mu$ ,  $\delta$  and  $\kappa$  receptors, on food intake and food choice.

## 11.3 MATERIALS AND METHODS

### 11.3.1 *Experimental animals, diets and drugs*

Adult male *S. crassicaudata* housed under a long-day (16-h light, 8-h dark) light regime, lights turned off at 15:30h, were used. Standard laboratory diet was provided *ad libitum*, except where stated. Mealworms were provided *ad libitum* where stated. Data for food intake are expressed as kcal/g of body weight or total kcal eaten.

The opioid antagonists naloxone, naltrindole, and nor-BNI were prepared and administered as outlined in section 3.5.4.

### 11.3.2 *Experimental protocols*

#### Study 1: Food choice in *S. crassicaudata*; mealworms vs standard laboratory diet

To determine whether *S. crassicaudata* prefer mealworms or their standard laboratory diet, 11 *S. crassicaudata* were separated into two similar groups based on body weight. One group was fed only mealworms *ad libitum* for 26 days, the other was fed only standard laboratory diet *ad libitum* for 26 days. On day 26, all food was removed from cages at lights out. Animals were deprived of food for 24 hours, weighed, then re-fed with both mealworms and laboratory diet, with the intake of each food type being recorded at 0.5, 1, 2, 4, 6, and 24 hours.

#### Study 2: Effect of opioid antagonists on food intake

To determine the effects of the opioid antagonists naloxone, naltrindole and nor-BNI on deprivation-induced feeding of laboratory diet alone, two groups of *S. crassicaudata* with equal body weights ( $n = 7-16$  per group) were used. Food was removed from each cage at lights out (15:30h) on the day prior to the experiment. The next day animals were weighed and injected with either saline or one of the opioid antagonists naloxone (5, 10, 15 mg/kg), naltrindole (10, 15, 20 mg/kg) or nor-BNI (10, 15 mg/kg). At lights out, pre-weighed laboratory diet was introduced into the cage and subsequent food intake recorded. In animals injected with naloxone and naltrindole, food intake was recorded at 0.5, 1 and 2 hours following re-feeding. In animals injected with nor-BNI, food intake was recorded at 0.5, 1, 2, 4, 6 and 24 hours after re-feeding.

**Study 3: *Effect of opioid antagonists on food choice***

To determine the effect of the opioid antagonists naloxone, naltrindole and nor-BNI on food choice after 24-hour food deprivation, a protocol similar to that described in study 1 above was used, but the animals were re-fed with both mealworms and laboratory diet. The doses of naloxone were 0.5, 1, 5, 10 and 15 mg/kg. Doses of naltrindole and nor-BNI used were as in study 1.

**11.3.3      *Statistical analyses***

Statistical analyses were performed as outlined in section 3.9.

**11.4      RESULTS****11.4.1      Study 1: *Food choice in S. crassicaudata*; mealworms vs standard laboratory diet**

The animals fed only laboratory diet for 26 days had a lower body weight ( $13.04 \pm 0.34$  g) than animals fed only mealworms for 26 days ( $15.68 \pm 0.39$  g) ( $P < 0.01$ ). When offered a choice of unlimited laboratory diet and mealworms following a 24-hour fast, animals ate more mealworms than laboratory diet. This was so for both the animals that had received laboratory diet only for the 26 days prior to fasting ( $F_{3,95} = 23$ ,  $P < 0.0001$ ,  $n = 4$ ), and for those that had received mealworms only ( $F_{3,167} = 26.4$ ,  $P < 0.0001$ ,  $n = 7$ ), Fig 11.1. Total food intake (kcal/g body weight) during the 24-hour post-fast re-feeding period was greater in the animals previously fed laboratory diet than those fed mealworms ( $F_{1,65} = 6.46$ ,  $P = 0.030$ ). This was due to a greater intake of mealworms

( $F_{5,65} = 3.71$ ,  $P = 0.007$ ), with there being no difference in intake of laboratory diet between the two groups, Fig 11.1.

#### 11.4.2 Study 2: Effect of opioid antagonists on food intake

Although not measured directly, animals injected with naloxone, naltrindole and nor-BNI at the tested doses showed no major alteration in locomotor activity or other behaviours. When administered to animals fed laboratory diet only, naloxone at 10 mg/kg resulted in a 38% decrease ( $F_{2,53} = 3.33$ ,  $P = 0.049$ ,  $n = 10$ ), and at 15 mg/kg a 31% decrease ( $F_{2,89} = 3.28$ ,  $P = 0.045$ ,  $n = 16$ ) in food intake (kcal) in the first half hour after re-feeding when compared to control animals, while naloxone at 5 mg/kg ( $n = 10$ ) had no effect on food intake, Fig 11.2. Naltrindole, at the highest dose of 20 mg/kg, decreased food intake by 65% at 0.5-1 hours after re-feeding. There were significant drug ( $F_{1,89} = 4.18$ ,  $P = 0.046$ ), and drug x time effects ( $F_{2,89} = 5.23$ ,  $P = 0.007$ ,  $n = 16$ ). Naltrindole in doses of 10 mg/kg ( $n = 8$ ) and 15 mg/kg ( $n = 16$ ) had no effect on food intake at any time point, Fig 11.2. Nor-BNI at 10 mg/kg ( $n = 7$ ) and 15 mg/kg ( $n = 7$ ) also had no effect on food intake at any time point, Fig 11.2.

#### 11.4.3 Study 3: Effect of opioid antagonists on food choice

Naloxone suppressed food intake in the first 30 minutes after re-feeding, when administered to animals offered a choice of mealworms and laboratory diet. Total caloric intake was suppressed by 29% ( $F_{2,83} = 5.74$ ,  $P = 0.006$ ,  $n = 14$ ) by a naloxone dose of 1 mg/kg, 33% ( $F_{2,77} = 3.04$ ,  $P = 0.049$ ,  $n = 10$ ) by 5 mg/kg, 16% ( $F_{2,89} = 3.64$ ,  $P = 0.031$ ,  $n = 16$ ) by 10 mg/kg and 35% ( $F_{2,89} = 10.69$ ,  $P < 0.0001$ ,  $n = 16$ ) by 15 mg/kg, Fig 11.3. This contrasts with the higher doses of naloxone ( $\geq 10$  mg/kg) that were needed to

suppress food intake in animals re-fed with laboratory diet alone (see study 2). The greater appetite-suppressing effect of naloxone in animals fed the mixed diet was due to a preferential suppression of mealworm intake. Naloxone at 1, 5, 10 and 15 mg/kg resulted in reductions of 30% ( $F_{2,83} = 6.09$ ,  $P = 0.004$ ), 41% ( $F_{2,77} = 3.61$ ,  $P = 0.03$ ), 40% ( $F_{2,119} = 3.95$ ,  $P = 0.023$ ), and 41% ( $F_{2,89} = 15.90$ ,  $P < 0.0001$ ) respectively, in intake of mealworms, whereas 0.5 mg/kg ( $n = 14$ ) had no effect, Fig 11.4. There was a compensatory increase in mealworm intake in the 30-60 minute period of re-feeding with a dose of 15 mg/kg ( $P < 0.05$ ), Fig 11.4. In contrast, intake of laboratory diet was not affected by naloxone at any time point, Table 11.1. Naltrindole, in doses of 10 mg/kg ( $n = 7$ ), 15 mg/kg ( $n = 7$ ) and 20 mg/kg ( $n = 15$ ), had no effect on the intake of either mealworms, Fig 11.4, or laboratory diet (data not shown) in the first 2 hours after re-feeding. Nor-BNI in doses of 10 mg/kg ( $n = 7$ ) and 15 mg/kg ( $n = 7$ ) had no significant effect on intake of either mealworms or laboratory diet (data not shown).

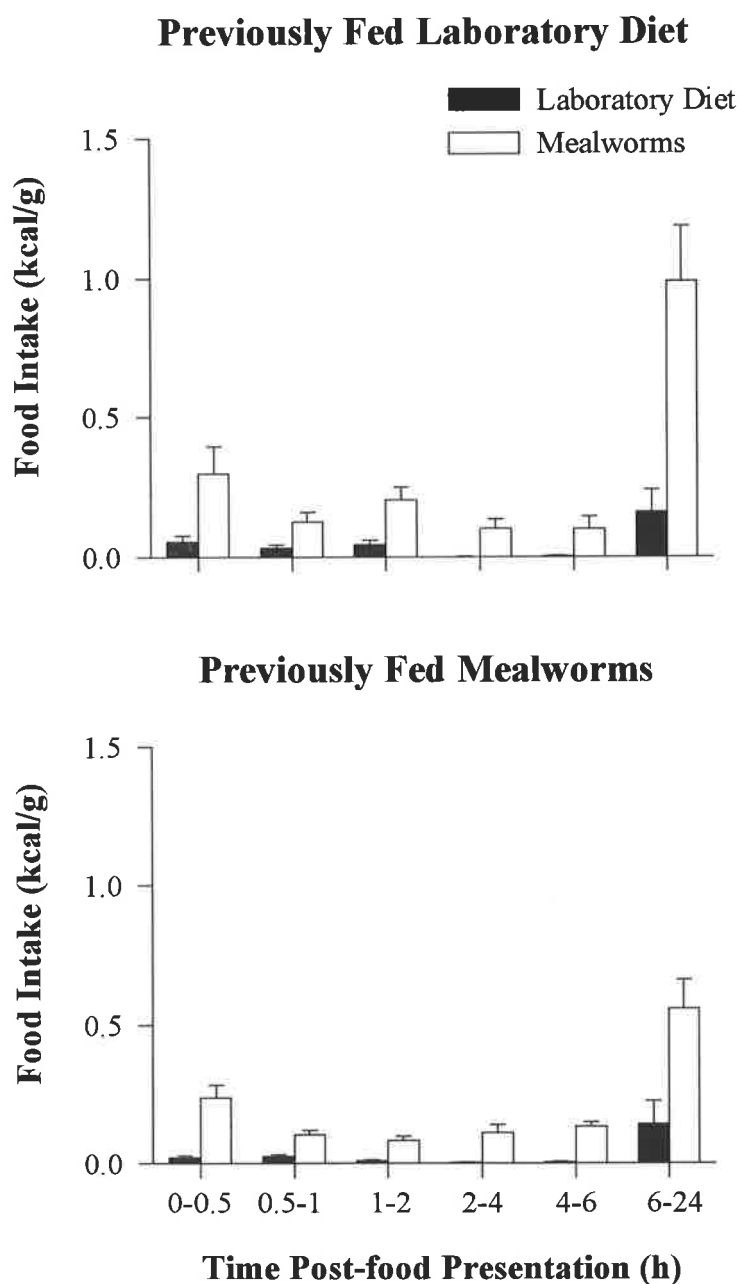


**Figure 11.1:** Energy intake (kcal/g body weight) of laboratory diet and mealworms in 24-hour fasted male *S. crassicaudata* previously fed laboratory diet only (upper panel) or mealworms only (lower panel), for 26 days. Both groups of animals subsequently consumed mealworms in preference to laboratory diet,  $P < 0.0001$ . Data are mean  $\pm$  SE.

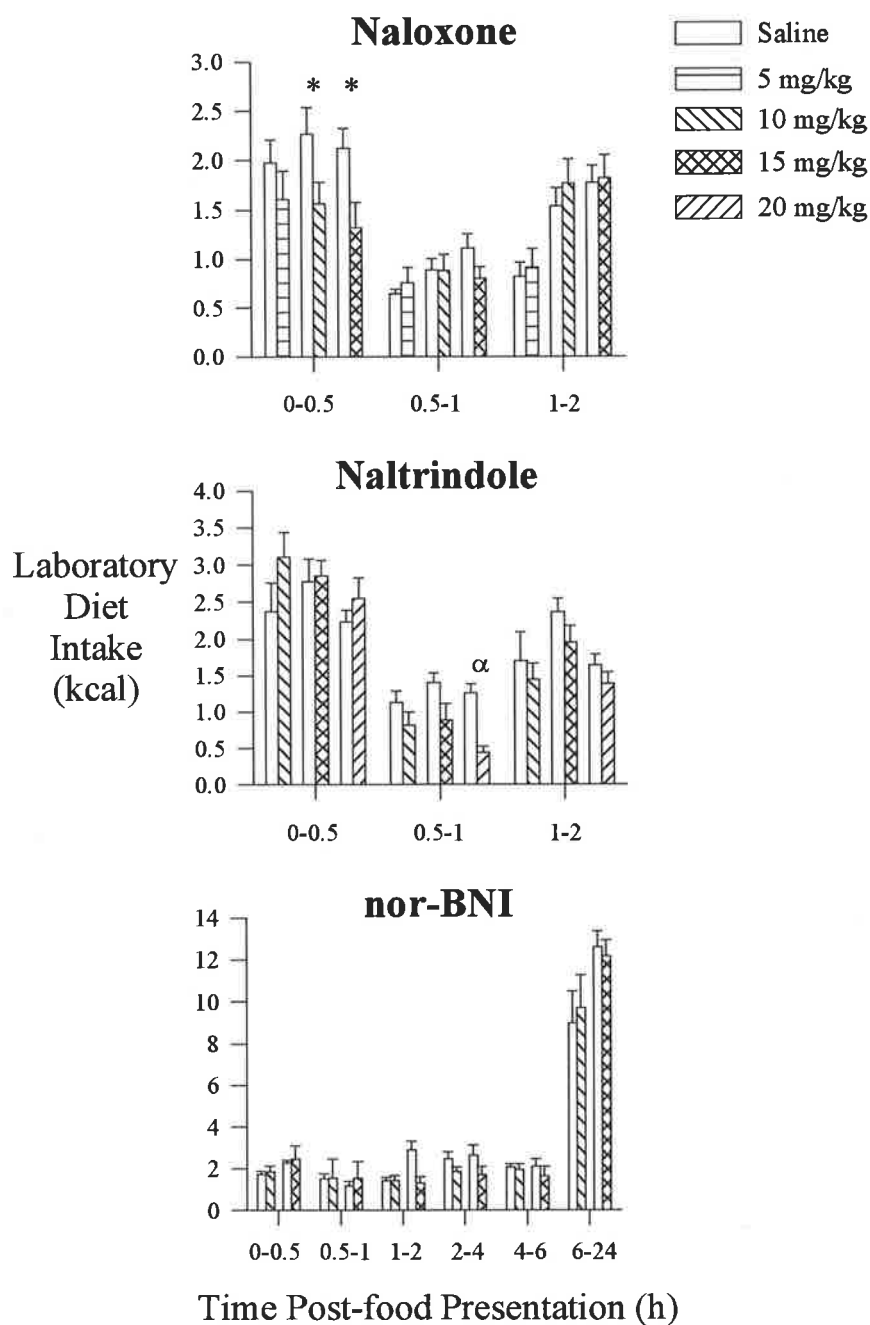


**Figure 11.2:** Effects of naloxone, naltrindole and nor-BNI on intake of laboratory diet after food deprivation in male *S. crassicaudata* offered laboratory diet alone. Naloxone in doses of 10 and 15 mg/kg reduced intake in the first 30 minutes after re-feeding. Naltrindole at a dose of 20 mg/kg reduced intake at 30-60 minutes after re-feeding. Nor-BNI had no effect ( $\alpha$   $P < 0.001$ , \*  $P < 0.05$ ). Data are mean  $\pm$  SE.

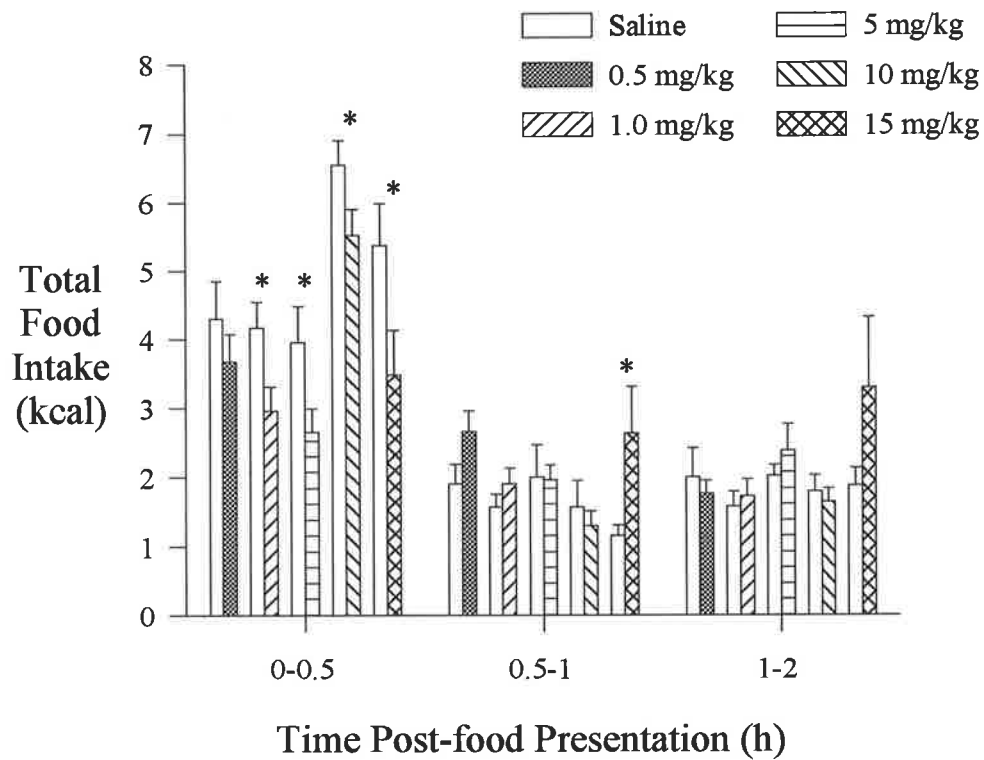


**Figure 11.3:** Effect of naloxone on total caloric intake (kcal), following a 24-hour fast in male *S. crassicaudata* offered a choice of laboratory diet and mealworms. Doses of 1, 5, 10, and 15 mg/kg reduced total caloric intake in the 0-30 minute period after re-feeding (\*  $P < 0.05$ ). Data are mean  $\pm$  SE.



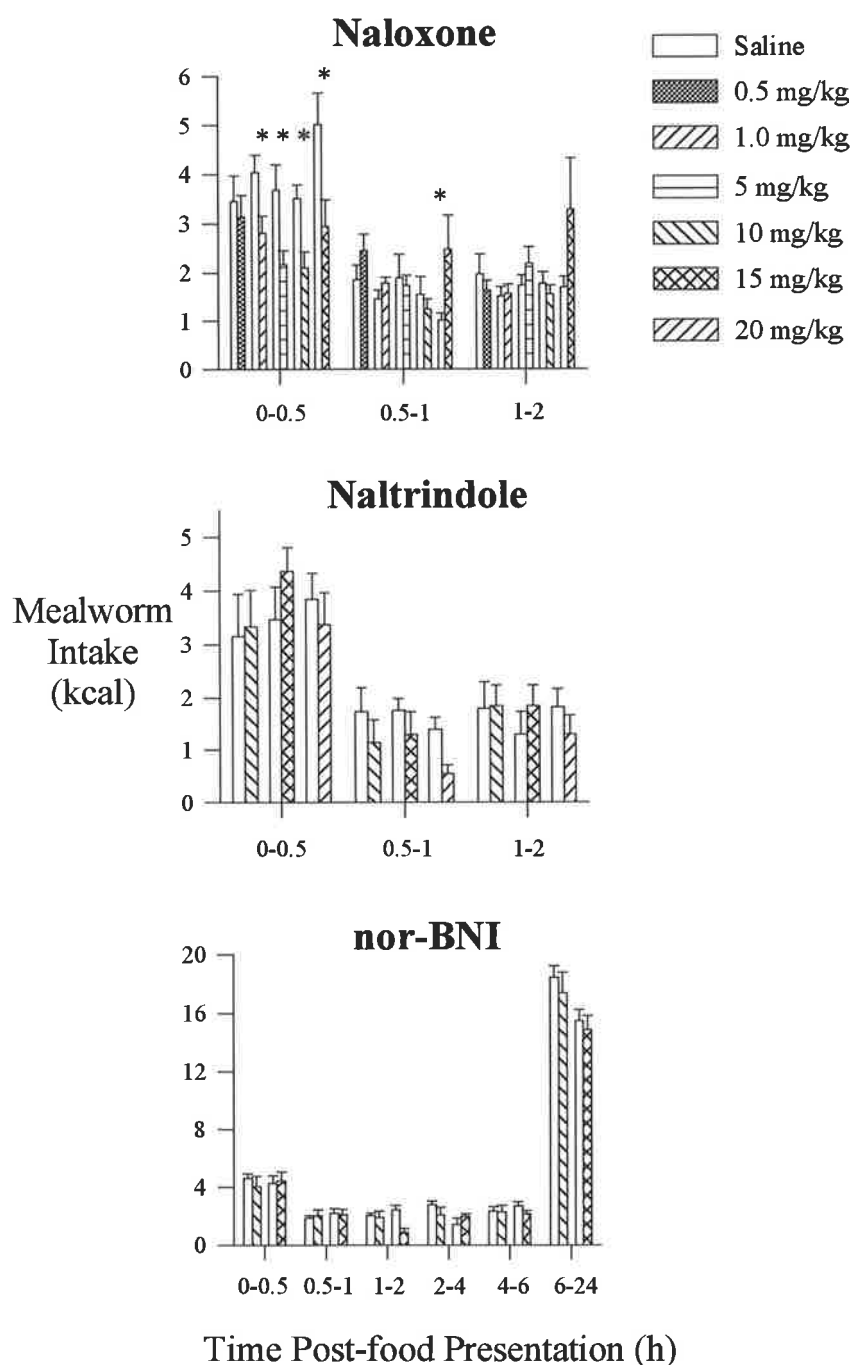


**Figure 11.4:** Effects of naloxone, naltrindole and nor-BNI on intake of mealworms in food-deprived male *S. crassicaudata* offered a choice of laboratory diet and mealworms. Naloxone in doses of 1, 5, 10, and 15 mg/kg reduced intake in the first 30 minutes after re-feeding. Naltrindole and nor-BNI had no effect (\*  $P < 0.05$ ). Data are mean  $\pm$  SE.

| <b>TREATMENT</b>          | <b>0-30 min</b> | <b>30-60 min</b> | <b>60-120 min</b> |
|---------------------------|-----------------|------------------|-------------------|
| <b>Saline</b>             | 0.84 ± 0.19     | 0.06 ± 0.04      | 0.02 ± 0.01       |
| <b>Naloxone 0.5 mg/kg</b> | 0.52 ± 0.12     | 0.21 ± 0.17      | 0.15 ± 0.14       |
| <b>Saline</b>             | 0.13 ± 0.06     | 0.12 ± 0.05      | 0.08 ± 0.04       |
| <b>Naloxone 1 mg/kg</b>   | 0.17 ± 0.05     | 0.12 ± 0.04      | 0.17 ± 0.06       |
| <b>Saline</b>             | 0.27 ± 0.12     | 0.10 ± 0.04      | 0.23 ± 0.21       |
| <b>Naloxone 5 mg/kg</b>   | 0.48 ± 0.18     | 0.23 ± 0.07      | 0.19 ± 0.06       |
| <b>Saline</b>             | 0.42 ± 0.13     | 0.03 ± 0.01      | 0.02 ± 0.01       |
| <b>Naloxone 10 mg/kg</b>  | 0.42 ± 0.13     | 0.06 ± 0.02      | 0.10 ± 0.03       |
| <b>Saline</b>             | 0.36 ± 0.13     | 0.15 ± 0.07      | 0.19 ± 0.16       |
| <b>Naloxone 15 mg/kg</b>  | 0.54 ± 0.21     | 0.15 ± 0.14      | 0.01 ± 0.01       |

**Table 11.1:** Effect of naloxone on intake of laboratory diet (kcal) in food deprived male *S. crassicaudata* offered a choice of laboratory diet and mealworms. There was no difference in laboratory diet intake between controls and naloxone treated animals. Data are mean ± SE.

## 11.5 DISCUSSION

This study has shown that *S. crassicaudata* exhibits opioid modulation of both food intake and food choice; the first evidence for a role of endogenous opioid peptides in the regulation of appetite in marsupials. In *S. crassicaudata*, this regulation appears to be mediated predominantly by the  $\mu$  opioid receptor, with the  $\delta$  receptor playing at most a minor role. Naloxone is a short acting antagonist and, accordingly, maximal effects on food intake are evident soon after administration (Levine *et al* 1995, Marks-Kaufman & Kanarek 1981). In both rats (Levine *et al* 1995), and now in *S. crassicaudata*, naloxone has been shown to suppress food intake during the first 30 minutes after its administration.

Although naloxone has similar effects in rats and *S. crassicaudata*, the role of other opiate receptor subtypes in appetite regulation appears to differ between the two species. Naloxone is infact not a pure  $\mu$  opioid receptor antagonist, and has some ability to antagonise  $\delta$  opioid receptors. Naloxone, however, had a greater effect on appetite in *S. crassicaudata* than either naltrindole, which is a specific  $\delta$  antagonist, or nor-BNI, which is a specific  $\kappa$  antagonist. The lack of a major effect of  $\delta$  and  $\kappa$  antagonists on appetite in *S. crassicaudata* supports the concept of a specific  $\mu$  effect on feeding in this species. In rats both  $\kappa$  and  $\mu$  receptor subtypes appear to be important in appetite and food choice. Romsos *et al* (1987) found that the  $\kappa$  agonist butorphanol tartrate was a more effective stimulator of feeding in rats than ketocyclazocine which is predominantly, but not specifically, a  $\kappa$  agonist. In rats, nor-BNI has been shown to decrease intake of a high fat

diet (Arjune & Bodnar 1990), and dynorphin, the endogenous  $\kappa$  ligand, is a potent stimulator of food intake in rats (Morley *et al* 1982b, 1983c).

Naltrindole is reported to be the only highly selective  $\delta$  antagonist that is active after peripheral administration (Portoghese *et al* 1988). A high dose of 20 mg/kg was required to decrease food intake in *S. crassicaudata*, and this decrease only occurred in the second 30 minutes after re-feeding with laboratory diet. In previous studies it has been suggested that the  $\delta$  receptor plays only a minor role in appetite behaviour, however DSLET, a selective  $\delta$  agonist, stimulates feeding in mice when given intracerebroventricularly (Gosnell *et al* 1986).

This study shows that *S. crassicaudata* clearly prefer mealworms to their usual laboratory diet, and this occurs even when the novelty of the preferred food is eliminated. In all experiments involving a food choice, animals ate predominantly mealworms. These data support previous findings that the anorectic effects of naloxone are greater when animals are fed a preferred or more palatable food (Levine *et al* 1982b, 1995, Marks-Kaufman & Kanarek 1981). When offered a choice of laboratory diet and the more palatable mealworms, the dose of naloxone required to decrease intake of the mealworms was 1.0 mg/kg; ten fold less than that required to reduce intake when offered laboratory diet alone. The protein (60%) and fat (30%) content of mealworms is higher than the laboratory diet, which contains 25% protein and 20% fat by dry weight. These findings are therefore compatible with observations in rats showing that naloxone decreases intake of a high fat diet to a greater extent than a high carbohydrate diet (Romsos *et al* 1987).

The observations that neither naltrindole nor nor-BNI affected food choice in *S. crassicaudata* also suggests that in *S. crassicaudata* the  $\mu$  opioid receptor plays the major role in both food intake and choice of a preferred food. Studies in rats suggest that morphine, which is predominantly a  $\mu$  agonist, increases intake of a preferred diet rather than intake of a specific macronutrient (Gosnell *et al* 1990). There is also evidence that intake of palatable foods actually stimulates the release of opioids such as beta endorphin, resulting in overeating and increased weight (Dum *et al* 1983, Mandenoff *et al* 1982). This effect is mediated through the  $\mu$  opioid receptor pathway and hence supports a major  $\mu$  opioid receptor mediated effect on feeding.

Nor-BNI, a highly selective  $\kappa$  antagonist (Portoghese *et al* 1987), reduces feeding for up to 24 hours when administered intracerebroventricularly to 24-hour food-deprived rats (Levine *et al* 1990). The magnitude of these effects are, however, relatively modest (28% decrease after 100 nmol) and independent of the time of administration in relation to the availability of food (*ie.* immediate, 1 or 2 hours prior). There is a lack of consensus in relation to both the pre-treatment times and the duration of action of nor-BNI (Jones & Holtzman 1992). Endoh *et al* (1992) used the tail pinch test in mice and found that the antagonistic action of nor-BNI plateaued at 2 hours and continued for 4 days. In contrast, when administered intracerebroventricularly in rats, a 10 minute pre-treatment time is sufficient to suppress intake of a sweet solution (Calcagnetti *et al* 1989). Following an examination of the literature, and after performing pilot studies using no pre-treatment time and 2 hours pre-treatment time, a pre-treatment time of 2 hours was employed in the studies using nor-BNI. At the doses tested, nor-BNI had no

effect on food intake or food choice. Furthermore, in studies using no pre-treatment time, nor-BNI also had no effect on food intake or choice.

The possibility that naloxone may have had a non specific effect on food intake cannot be totally discounted. Studies in humans have shown that naloxone decreases food intake without altering perception of hunger (Trenchard & Silverstone 1983). In wolves (Morley *et al* 1983b) and tigers (Billington *et al* 1985), higher doses of naloxone are associated with regurgitation and vomiting in some animals. There was no evidence of illness in *S. crassicaudata* after any of the doses of naloxone. Furthermore, if non-specific effects of naloxone were responsible for these observations, it may have been expected that a reduction in laboratory diet intake would have been evident with doses that reduced intake of the preferred food.

These studies have shown that endogenous opioids modulate feeding in the marsupial *S. crassicaudata*. Marsupials thus join the wide variety of species including molluscs, birds and many mammals, in which such effects occur. This conservation of opioid mediated feeding across such a wide variety of species supports the concept that endogenous opioids are of fundamental importance to the regulation of appetite. In *S. crassicaudata* these effects seem to be mediated predominantly by the  $\mu$  opioid receptor, demonstrating the importance of food palatability in modulating the effectiveness of the opioid feeding system.

## CHAPTER 12

**GASTROINTESTINAL PEPTIDES, PHOTOPERIOD, AND THE  
REGULATION OF FOOD INTAKE IN *S. CRASSICAUDATA*****12.1 SUMMARY**

Peptides released from the gastrointestinal tract in response to food intake have been demonstrated to decrease feeding in mammals, although these effects may be species specific and photoperiod dependent. The effect of gastrointestinal peptides, other than urocortin (see chapter 11), on feeding has not previously been determined in marsupials. The aim of this study was to examine the effect of peripherally administered cholecystokinin (CCK), bombesin (BBS), gastrin releasing peptide (GRP) and glucagon like peptide-1 (GLP-1) on food intake in *S. crassicaudata*. Male *S. crassicaudata* ( $n = 7-15$  per group) were deprived of food for 24 hours and then injected intraperitoneally with either saline, CCK-8 (5, 10, 20, 40, 60  $\mu\text{g/kg}$ ), BBS (10, 15, 20, 40  $\mu\text{g/kg}$ ), GRP (20, 60  $\mu\text{g/kg}$ ) or GLP-1 (0.1, 1, 2  $\text{mg/kg}$ ). Animals were then fed laboratory diet *ad libitum* and subsequent food intake was determined at 0.5, 1, 2, 3 and 24 hours. The experiment was performed in both long-day (LD; 16-h light, 8-h dark) and short-day (SD; 8-h light, 16-h dark) housed animals. Only the highest doses of each peptide was tested in the SD housed animals.

Peripherally administered CCK, BBS and GLP-1 had no acute effects on food intake in *S. crassicaudata* housed under either LD or SD photoperiods, at any of the doses tested. By contrast, GRP (60 µg/kg) induced a small decrease in food intake in SD, but not LD housed animals. This study provides the first information regarding the effects of peripheral administration of the gastrointestinal peptides CCK, BBS, GRP and GLP-1 on food intake in a marsupial species, and provides further evidence that substantial differences exist between species in the feeding response to these peptides.

## 12.2 INTRODUCTION

The physiological mechanisms that produce satiety following food intake are complex and still not fully understood. Numerous peptides localised within the gastrointestinal tract and central nervous system (CNS) are released in response to feeding, and several of these have been shown to suppress food intake when administered peripherally to rats and mice (Morley 1987, 1990, 1995, Woods *et al* 1981). It has been proposed that some of these peptides function as regulatory signals to terminate feeding (Morley *et al* 1985a). The gastrointestinal peptide that has been studied most extensively is cholecystokinin (CCK), (see section 1.6.3.2), however other peptides such as bombesin (BBS) (see section 1.6.6.3) and the closely related gastrin releasing peptide (GRP) (see section 1.6.6.3) have also been demonstrated to decrease feeding. Glucagon-like peptide 1 (GLP-1) (see section 1.6.3.4) reduces food intake in lean humans when infused intravenously at 50 pmol/kg/h (Flint *et al* 1998). GLP-1, however, fails to decrease feeding in rats when administered peripherally at doses of



up to 500  $\mu\text{g}$  (Turton *et al* 1996). However, intracerebroventricular GLP-1 (10-100  $\mu\text{g}$ ) decreases feeding in a dose dependent manner in rats (Turton *et al* 1996).

To date, the majority of studies examining peripheral effects of gastrointestinal peptides on food intake have been performed in rats and mice. There is evidence, however, suggesting that the effects of these peptides on food intake are species specific (Bartness *et al* 1986, Morley *et al* 1983a,c). For example, in contrast to rats, the Siberian (*Phodopus sungorus*) and Chinese (*Cricetulus griseus*) hamsters fail to decrease food intake in response to peripheral administration of the pancreatic peptide glucagon (1 mg/kg) (Bartness *et al* 1986, Billington *et al* 1984), and both CCK (40  $\mu\text{g/kg}$ ) and BBS (40  $\mu\text{g/kg}$ ) fail to effect food intake in Chinese hamsters (Billington *et al* 1984). In addition, the effect of peripherally administered peptides on satiety may be dependent on photoperiod (Bartness *et al* 1986). For example, the Siberian hamster is more sensitive to CCK and BBS administration when housed on short- as opposed to long-day photoperiods (Bartness *et al* 1986). Siberian hamsters decrease their body weight and carcass lipid on short-day photoperiods (Vitale *et al* 1985, Wade & Bartness 1984a), suggesting heightened sensitivity to satiety peptides may be involved in the decreased food intake associated with these changes.

Studies presented in this thesis have demonstrated that endogenous opioid peptides (see chapter 11), and the hypothalamic peptide CRF (see chapter 10), decrease food intake in *S. crassicaudata* following peripheral administration. In addition, the peptide urocortin, which is abundantly expressed in the gastrointestinal tract, has also been demonstrated to decrease food intake in *S. crassicaudata* (see chapter 10). However,

the effects of other peptides released from the gastrointestinal tract on food intake have not previously been determined in a marsupial. The sequence of CCK-8 is completely conserved across Old World mammals (see Eng *et al* 1992), and differs by only one amino acid substitution in the guinea pig and chinchilla (Fan *et al* 1987, Zhou *et al* 1985). CCK-8 sequences from the opossum (*Monodelphis domestica*), eastern quoll (*Dasyurus viverrinus*) and tammar wallaby (*Macropus eugenii*) are identical to that of Old World mammals (Eng *et al* 1992, Fan *et al* 1988), and CCK binding sites are present in the Brazilian opossum brain (Kuehl-Kovarik *et al* 1993). It is therefore likely that CCK developed before marsupials diverged from other mammals. GLP-1 similarly has been shown to be completely conserved in all mammalian species studied to date (Orskov 1992), and GLP-1 levels in plasma from the marsupial *S. crassicaudata* can be measured using an antibody to human GLP-1 (GA Wittert, personal communication). High levels of sequence conservation imply that these peptides may play a critical physiological role, and therefore are likely to be involved in regulation of food ingestion in marsupials. The aim of this study was to determine the effect of the gastrointestinal peptides CCK-8, BBS, GRP and GLP-1 on food intake in both long- and short-day housed *S. crassicaudata*.

## 12.3 MATERIALS AND METHODS

### 12.3.1 *Experimental animals, diets and drugs*

Adult male *S. crassicaudata* were studied. Animals were housed under either a long-day (LD; 16-h light, 8-h dark) or a short-day (SD; 8-h light, 16-h dark) light regime,

lights turned off at 16:30h. Laboratory diet was provided *ad libitum* except where stated. Food intake was expressed as kcal.

CCK-8, BBS, GRP and GLP-1 were prepared as stated in section 3.5.5. 0.9% saline, CCK-8, BBS, GRP, and GLP-1 were administered intraperitoneally (IP). Initial doses of CCK, BBS and GRP used were based on those previously shown to be effective in rats (Gibbs 1985, Silver *et al* 1989). The initial dose of GLP-1 used was based on a dose previously tested in rats (Turton *et al* 1996).

### 12.3.2 *Experimental protocols*

To determine the effect of CCK-8, BBS, GRP and GLP-1 on food intake, groups of *S. crassicaudata* with similar body weights were studied ( $n = 7-15$  per group). Following a period of 24-hour food deprivation, animals were injected at the onset of the dark phase with either saline, CCK-8 (5, 10, 20, 40, 60  $\mu\text{g/kg}$ ), BBS (10, 15, 20, 40  $\mu\text{g/kg}$ ), GRP (20, 60  $\mu\text{g/kg}$ ), or GLP-1 (0.1, 1.0, 2.0  $\text{mg/kg}$ ). *S. crassicaudata* were subsequently re-fed *ad libitum* with standard laboratory diet, and food intake was recorded at 0.5, 1, 2, 4, 6, and 24 hours.

The experiment was performed in both LD and SD housed animals, however only the highest dose of each drug was tested in each SD animal; CCK-8 (60  $\mu\text{g/kg}$ ), BBS (40  $\mu\text{g/kg}$ ), GRP (60  $\mu\text{g/kg}$ ), GLP-1 (2.0  $\text{mg/kg}$ ). Different drugs and doses were administered in a counterbalanced order in tests 7 days apart, such that no animal received more than one dose of drug within a two week period.

### 12.3.3 *Statistical analyses*

Data analyses were performed as described in section 3.9.

## 12.4 RESULTS

### 12.4.1 *The effect of CCK-8 on energy intake in S. crassicaudata*

In LD housed animals, neither CCK-8 5 µg/kg (drug  $F_{1,149} = 0.34$ ,  $P = 0.57$ , drug x time  $F_{4,149} = 0.77$ ,  $P = 0.54$ ), 10 µg/kg (drug  $F_{1,107} = 0.28$ ,  $P = 0.60$ , drug x time  $F_{4,134} = 0.78$ ,  $P = 0.54$ ), 20 µg/kg (drug  $F_{1,139} = 0.53$ ,  $P = 0.47$ , drug x time  $F_{4,139} = 0.001$ ,  $P = 1.0$ ), 40 µg/kg (drug  $F_{1,124} = 0.002$ ,  $P = 0.97$ , drug x time  $F_{4,124} = 0.08$ ,  $P = 0.99$ ), or 60 µg/kg (drug  $F_{1,124} = 0.006$ ,  $P = 0.94$ , drug x time  $F_{4,124} = 0.06$ ,  $P = 0.99$ ) had any effect on food intake at any time point as compared to saline treated controls,  $n = 12-15$ , Fig 12.1.

In SD housed animals, CCK-8 60 µg/kg (drug  $F_{1,99} = 2.44$ ,  $P = 0.14$ , drug x time  $F_{4,99} = 1.12$ ,  $P = 0.35$ ) had no effect on food intake at any time point as compared to the saline treated controls,  $n = 10$ , Fig 12.1.

### 12.4.2 *The effect of BBS on energy intake in S. crassicaudata*

In LD housed animals, neither BBS 10 µg/kg (drug  $F_{1,139} = 0.35$ ,  $P = 0.56$ , drug x time  $F_{4,139} = 0.23$ ,  $P = 0.92$ ), 15 µg/kg (drug  $F_{1,139} = 2.37$ ,  $P = 0.14$ , drug x time  $F_{4,139} = 0.99$ ,  $P = 0.41$ ), 20 µg/kg (drug  $F_{1,119} = 0.42$ ,  $P = 0.53$ , drug x time  $F_{4,119} = 0.39$ ,  $P = 0.82$ ), or 40 µg/kg (drug  $F_{1,119} = 0.50$ ,  $P = 0.49$ , drug x time  $F_{4,119} = 0.79$ ,  $P = 0.54$ )

had any effect on food intake at any time point as compared to saline treated controls,  $n = 12-14$ , Fig 12.2.

In SD housed animals, BBS 40  $\mu\text{g/kg}$  (drug  $F_{1,119} = 0.21$ ,  $P = 0.65$ , drug  $\times$  time  $F_{4,119} = 1.49$ ,  $P = 0.21$ ) had no effect on food intake at any time point as compared to the saline treated controls,  $n = 12$ , Fig 12.2.

#### 12.4.3 *The effect of GRP on energy intake in S. crassicaudata*

In LD housed animals, GRP 20  $\mu\text{g/kg}$  (drug  $F_{1,74} = 1.29$ ,  $P = 0.28$ , drug  $\times$  time  $F_{4,74} = 0.64$ ,  $P = 0.64$ ) and 60  $\mu\text{g/kg}$  (drug  $F_{1,149} = 0.26$ ,  $P = 0.61$ , drug  $\times$  time  $F_{4,149} = 0.167$ ,  $P = 0.95$ ) had no effect on food intake at any time point as compared to the saline treated controls,  $n = 7-15$ , Fig 12.3.

In contrast, GRP 60  $\mu\text{g/kg}$  administered to SD housed animals induced a decrease in food intake (drug  $F_{1,71} = 4.59$ ,  $P = 0.04$ ) as compared to the saline treated controls over the first 2 hours of the experiment, with significant differences observed at the 0.5 and 1-h time points,  $n = 12$ , Fig 12.3.

#### 12.4.4 *The effect of GLP-1 on energy intake in S. crassicaudata*

In LD housed animals, GLP-1 0.1  $\text{mg/kg}$  (drug  $F_{1,79} = 1.13$ ,  $P = 0.30$ , drug  $\times$  time  $F_{4,79} = 0.87$ ,  $P = 0.49$ ), 1  $\text{mg/kg}$  (drug  $F_{1,79} = 1.72$ ,  $P = 0.21$ , drug  $\times$  time  $F_{4,79} = 0.57$ ,  $P = 0.68$ ), and 2  $\text{mg/kg}$  (drug  $F_{1,129} = 1.58$ ,  $P = 0.22$ , drug  $\times$  time  $F_{4,129} = 1.10$ ,  $P = 0.36$ ) had no effect on food intake at any time point as compared to the saline treated controls,  $n = 8-13$ , Fig 12.4.

In SD housed animals, GLP-1 2 mg/kg (drug  $F_{1,87} = 0.94$ ,  $P = 0.34$ , drug x time  $F_{3,87} = 0.24$ ,  $P = 0.87$ ) had no effect on food intake at any time point in the first 4 hours of the experiment as compared to the saline treated controls. At 24 hours, however, food intake was increased in response to GLP-1 at 2 mg/kg ( $P = 0.04$ ),  $n = 11$ , Fig 12.4.

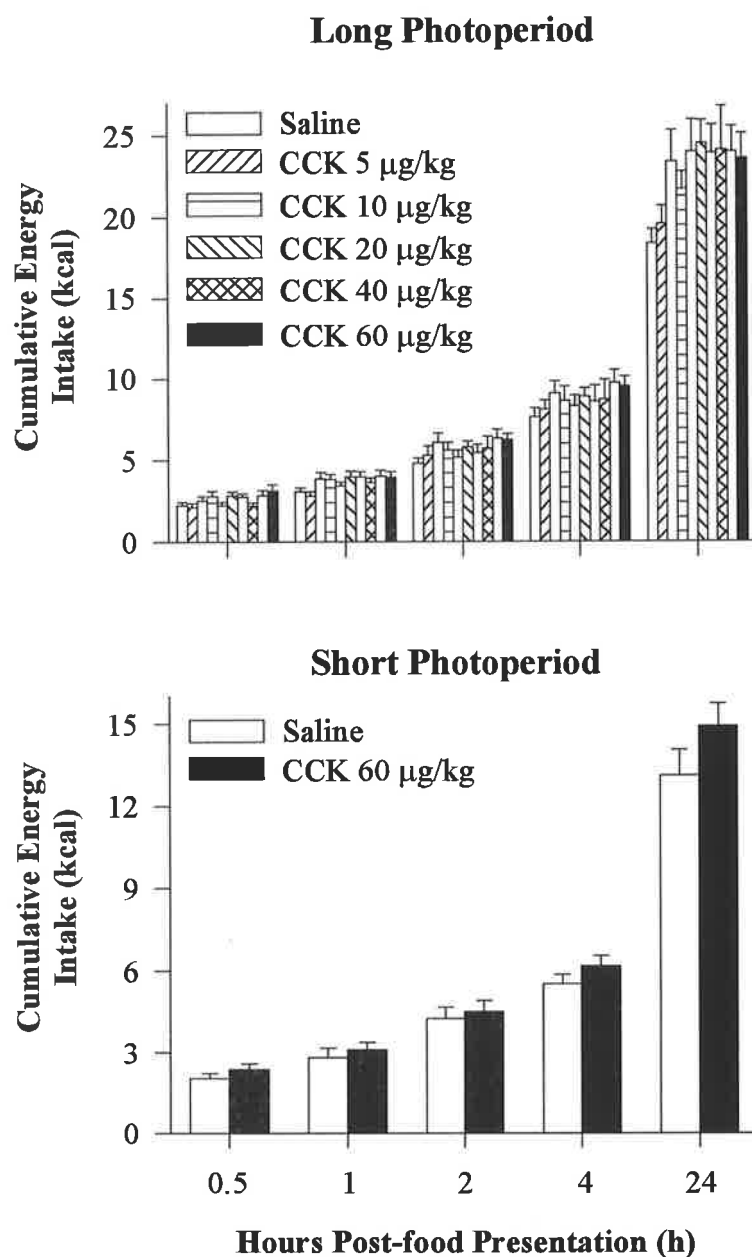


**Figure 12.1:** Cumulative energy intake (kcal) of 24-hour fasted male *S. crassicaudata* injected IP with saline or CCK-8 when housed under a LD (upper half) or a SD photoperiod (lower half). Subsequent food intake was measured at 0.5, 1, 2, 4, 6, and 24 hours. CCK was administered at 5, 10, 20, 40, and 60 µg/kg to LD, and at 60 µg/kg to SD animals. At the doses tested, CCK had no effect on food intake under either photoperiod. Values are mean  $\pm$  SE,  $n = 10-15$  per group.

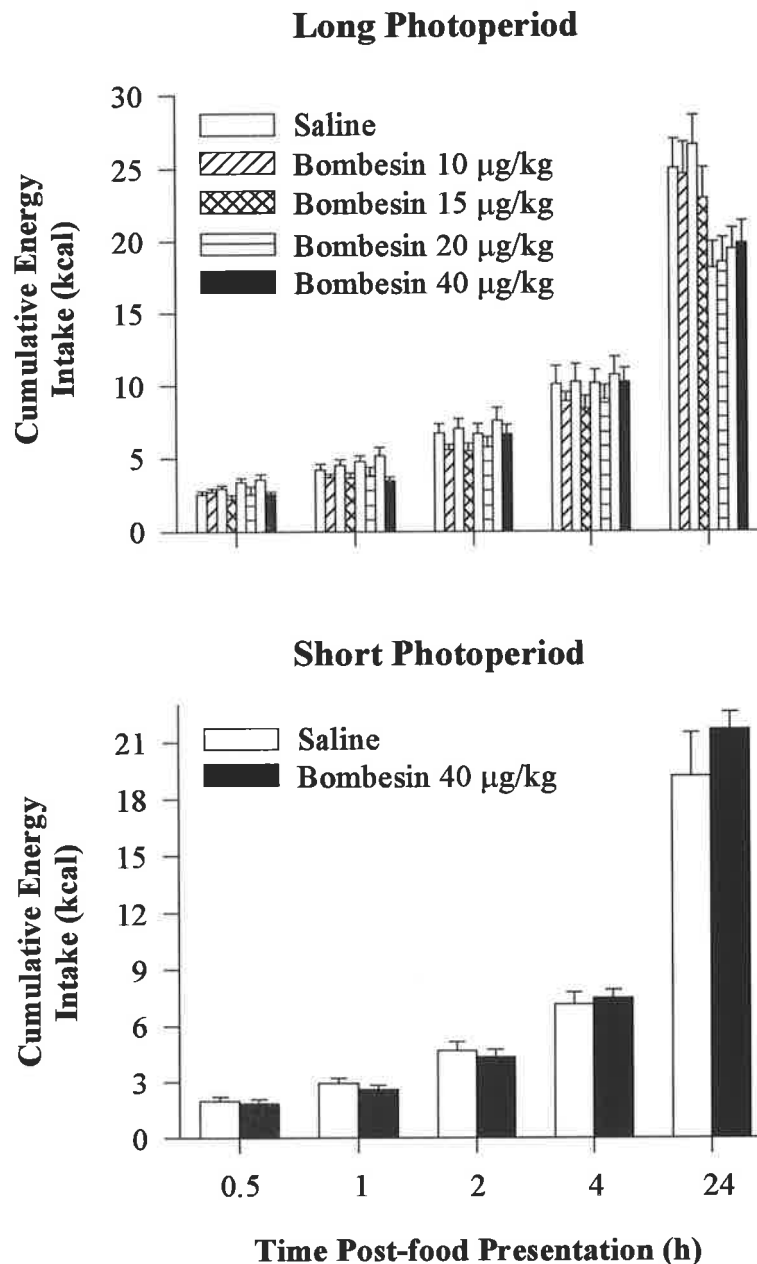


**Figure 12.2:** Cumulative energy intake (kcal) of 24-hour fasted male *S. crassicaudata* injected IP with saline or bombesin when housed under a LD (upper half) or a SD photoperiod (lower half). Subsequent food intake was measured at 0.5, 1, 2, 4, 6, and 24 hours. Bombesin was administered at 10, 15, 20, and 40 µg/kg to LD, and at 40 µg/kg to SD animals. At the doses tested, bombesin had no effect on food intake under either photoperiod. Values are mean  $\pm$  SE,  $n = 12-14$  per group.



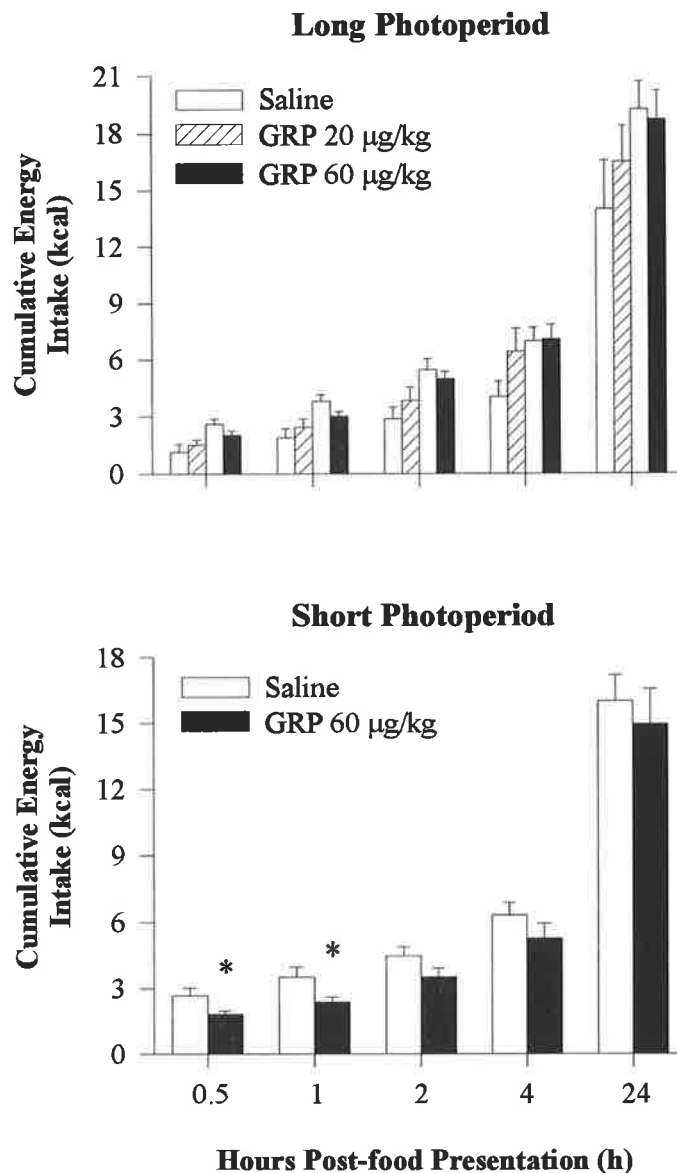


**Figure 12.3:** Cumulative energy intake (kcal) of 24-hour fasted male *S. crassicaudata* injected IP with saline or GRP when housed under a LD (upper half) or a SD photoperiod (lower half). Subsequent food intake was measured at 0.5, 1, 2, 4, 6, and 24 hours. GRP was administered at 20 and 60  $\mu\text{g/kg}$  to LD, and at 60  $\mu\text{g/kg}$  to SD animals. At the doses tested, GRP had no effect on food intake in animals housed under LD. Under a SD photoperiod, GRP at 60  $\mu\text{g/kg}$  induced a decrease in food intake over the first 2 hours, as compared to the saline treated controls. (\*  $P < 0.05$ ). Values are mean  $\pm$  SE,  $n = 7$ -15 per group.

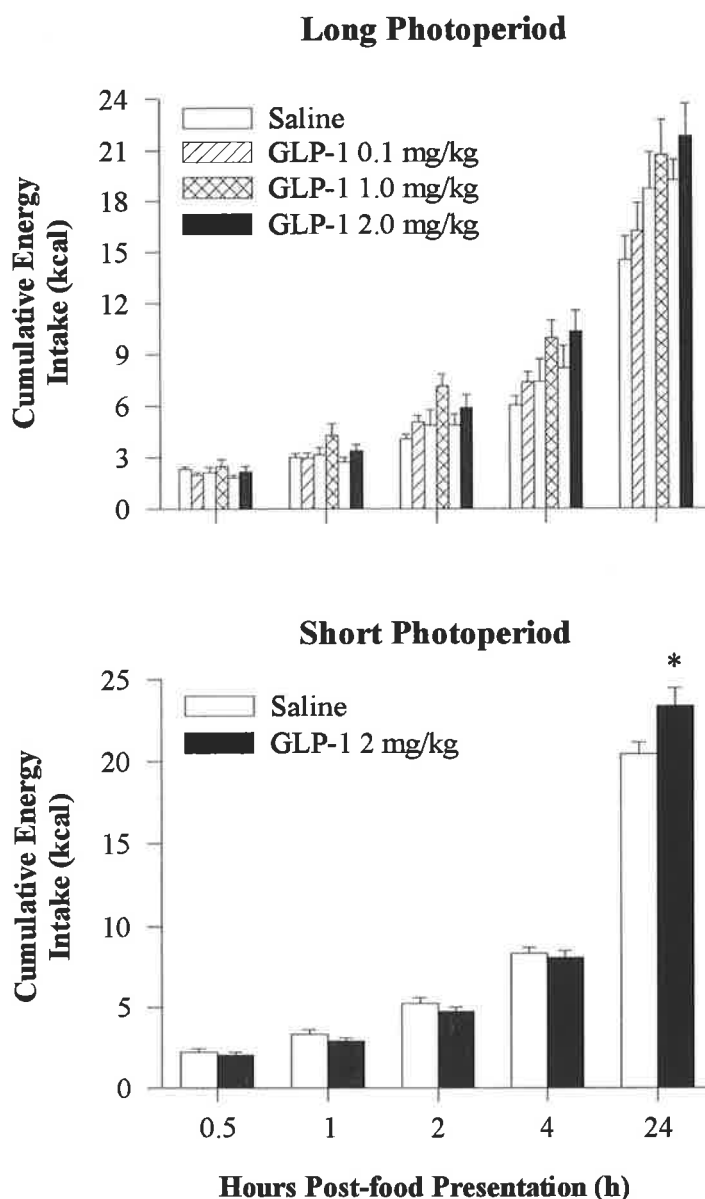


**Figure 12.4:** Cumulative energy intake (kcal) of 24-hour fasted male *S. crassicaudata* injected IP with saline or GLP-1 when housed under a LD (upper half) or a SD photoperiod (lower half). Subsequent food intake was measured at 0.5, 1, 2, 4, 6, and 24 hours. GLP-1 was administered at 0.1, 1.0, and 2.0 mg/kg to LD, and at 2.0 mg/kg to SD animals. At the doses tested, GLP-1 had no effect on food intake in animals housed under a LD photoperiod. GLP-1 at 2 mg/kg increased food intake in SD housed animals, but only at the 24 hour time point. (\*  $P = 0.04$ ). Values are mean  $\pm$  SE,  $n = 8-13$  per group.

## 12.5 DISCUSSION

This study has demonstrated that peripherally administered CCK up to 60  $\mu\text{g/kg}$  fails to induce a decrease in food intake in *S. crassicaudata* under either long- or short-day photoperiods. This finding is in contrast to that of many species including the dog, Siberian hamster and human, where peripheral CCK administration has been demonstrated to decrease food intake in doses as low as 0.1  $\mu\text{g/kg}$  (Bartness & Waldbillig 1984, Muurahainen *et al* 1988, Levine *et al* 1984). Bombesin and GLP-1 were similarly without any effect on food intake in *S. crassicaudata* at doses equivalent to or higher than those shown to be effective in other mammals. The bombesin-like peptide GRP, however, induced a decrease in food intake in short- but not long-day housed *S. crassicaudata* at a dose of 60  $\mu\text{g/kg}$ , which corresponds to GRP doses of 8  $\mu\text{g/kg}$  and 32  $\mu\text{g/kg}$  that were required to decrease food intake in baboons and rats respectively (Figlewicz *et al* 1985, Smith *et al* 1997).

The observation that peripherally administered CCK failed to effect food intake in a marsupial is of interest, as CCK has previously been shown to effectively decrease food intake in most species studied. CCK like immunoreactivity is detectable in the opisthobranch mollusc *Nivanax inermis* and CCK inhibits activity in the buccal ganglion neurons and the expansion of motoneurons which are responsible for prey capture (Zimering *et al* 1988). In goldfish (*Carassius auratus*), both central and peripheral administration of CCK inhibits food intake (Himick & Peter 1994), and CCK/gastrin-like immunoreactivity is evident in nuclear areas of the ventro-posterior hypothalamus and the hypothalamic inferior lobes (Himick & Peter 1994). In many

mammals including rats, dogs and humans, peripheral CCK administration has been shown to decrease food intake (Gibbs *et al* 1973, Kissileff *et al* 1981, Levine *et al* 1984, Morley *et al* 1985b). Taken together, these above studies suggest that gastrointestinal peptidergic regulation of feeding evolved early in the evolutionary process and has been remarkably well phylogenetically conserved. Nevertheless, differences in responsiveness to peripherally administered CCK have been demonstrated (Morley *et al* 1985b); some species such as the Chinese hamster, ground squirrel (*Spermophilus tridecemlineatus*) and wolf (*Canis lupus*) seem to be resistant to the actions of peripherally administered CCK to decrease food intake (Billington *et al* 1984, Morley *et al* 1983b, 1985b). One explanation may relate to the site of action of CCK; CCK's actions are predominantly peripheral in some animals such as rabbits, rhesus monkeys, Syrian hamsters, humans and dogs (see Morley *et al* 1985b for review), and at central sites in others such as sheep, pigs and chickens (Baile *et al* 1983, Denbow & Myers 1982, Parrott & Baldwin 1981). In rats under 'normal' feeding conditions, CCK produces its effect through a peripheral mechanism, via the activation of gastric vagal fibers (Morley *et al* 1982c, Smith *et al* 1981a). In contrast, with stress induced eating, CCK appears to decrease feeding through a central mechanism (Levine & Morley 1981b). Unlike in rats, peripheral CCK decreases food intake in dogs following vagotomy (Levine *et al* 1984). Species differences must therefore be taken into account when examining the effects of peripherally administered peptides on food intake.

The failure of acute peripheral GLP-1 administration to effect feeding in *S. crassicaudata* is consistent with observations in rats where doses of GLP-1 up to 500

$\mu\text{g}$  have no effect on food intake (Turton *et al* 1996). In addition, fragments of GLP-1 have been demonstrated to be inactive peripherally in isolated perfused rat pancreas (Suzuki *et al* 1989) and the GLP-1 knockout mouse shows no alterations in food intake compared to wild type mice (Scrocchi *et al* 1996). In humans, GLP-1 is secreted primarily in response to glucose or mixed meals, and is secreted in proportion to the caloric load delivered, providing a possible mechanism whereby the quantity of nutrients ingested could be signalled (Lavin *et al* 1998, Schirra *et al* 1996). In humans, peripheral GLP-1 has been demonstrated to decrease food intake in normal weight and obese humans (Drucker 1998). Therefore, despite the structural conservation of GLP-1, the actions of this peptide, at least peripherally, may vary between species.

Although bombesin and CCK have been demonstrated to have similar effects on feeding and pancreatic enzyme release, there is evidence that they act separately, and on different receptors (Basso *et al* 1975, Peikin *et al* 1979). Peripheral bombesin administration decreases food intake in rats, cats, wolves and humans (Bado *et al* 1989, Gibbs *et al* 1979, Morley *et al* 1986, Muurahainen *et al* 1983), with doses as low as 3  $\mu\text{g}/\text{kg}$  being effective at decreasing food intake in rats. In rats, peripherally administered bombesin has been demonstrated to decrease food intake by a mechanism that is not dependent on an intact vagus (Stuckey *et al* 1985). In the Siberian hamster, bombesin (up to 40  $\mu\text{g}/\text{kg}$ ) fails to effect food intake in long-day housed animals, but following short-day exposure bombesin (10  $\mu\text{g}/\text{kg}$ ) decreases food intake (Bartness *et al* 1986). In contrast, bombesin up to 40  $\mu\text{g}/\text{kg}$  has no effect on food intake in Chinese hamsters (Billington *et al* 1984).

Unlike bombesin and CCK, the physiological and behavioural actions of GRP have not been extensively studied. GRP is the mammalian analogue of bombesin, and has been demonstrated to acutely inhibit feeding in rats, baboons and humans following peripheral administration (Figlewicz *et al* 1985, Gutzwiller *et al* 1994, Stein & Woods 1983). In rats, GRP (32 µg/kg) inhibits feeding, however GRP has been demonstrated to be slightly less effective at reducing food intake on a molar basis than BBS (Smith *et al* 1997, Stein & Woods 1983). At the doses tested in this study, GRP did not decrease food intake in long-day housed *S. crassicaudata*, but significantly decreased food intake in short-day housed animals. This is the first demonstration that peripheral administration of a gastrointestinal peptide other than urocortin (see chapter 10) can affect food intake in a marsupial. The finding that GRP had no effect on food intake of long-day *S. crassicaudata* is consistent with studies in Siberian hamsters showing that sensitivity to CCK-8 and BBS is increased under short-day photoperiods (Bartness *et al* 1986). Interestingly, in contrast to *S. crassicaudata*, Siberian hamsters decrease body weight, carcass lipid and food intake under short-day photoperiods. The reason why Siberian hamsters reduce food intake under short-day photoperiods is unknown, but it may be the result of heightened sensitivity to factors that normally terminate food intake, such as putative satiety peptides (Bartness *et al* 1986).

There is some controversy in the literature as to whether the effect of gastrointestinal peptides, such as CCK and bombesin, to reduce food intake represents a true satiating effect or is secondary to toxicity and aversion. The majority of studies, however, support a true satiating effect of most peptides (see Morley *et al* 1985b). The effect of

peripheral urocortin and corticotrophin releasing factor to decrease food intake in *S. crassicaudata* (see chapter 10) did not appear to be the result of illness, as no change in oxygen consumption ( $VO_2$ ) was observed. Studies performed in this laboratory have previously demonstrated that unwell *S. crassicaudata* show a reduction in  $VO_2$  (Clements *et al* 1998). Although we did not measure  $VO_2$  in this study, it is unlikely that the observed effect of GRP to decrease food intake in *S. crassicaudata* housed under short-days was the result of aversion. Presumably if this was the case, a similar effect would have been observed in the long-day housed animals.

This study provides further evidence that substantial differences exist between species in the feeding response to peripherally administered gastrointestinal peptides, despite substantial structural conservation. In addition, alterations in photoperiod may significantly affect the food intake response to these peptides.

## CHAPTER 13

**BROWN FAT, UNCOUPLING PROTEINS AND MECHANISMS  
FOR ENERGY BALANCE IN *S. CRASSICAUDATA*****13.1 SUMMARY**

The presence of brown adipose tissue (BAT) in marsupials is controversial because attempts to identify mitochondrial uncoupling protein-1 (UCP1) have been unsuccessful. *S. crassicaudata* has an interscapular pad of adipose tissue, and electronmicroscopy has revealed this tissue to exhibit characteristics typical of BAT. Guanosine di-phosphate binding was present, and UCP1 protein was detected by immunoblot, confirming this tissue to be BAT. UCP1 mRNA was also detected by Northern blot analysis. Expression of UCP1 protein was increased by cold exposure, however there was no evidence that BAT metabolism was involved in re-warming from torpor or in maintaining body temperature during cold exposure.

*S. crassicaudata* are heterothermic; body temperature decreased on exposure to cold and increased again when ambient temperature was restored. Provided adequate food was available, *S. crassicaudata* did not enter torpor on prolonged exposure to low ambient temperature (15 °C). These data suggest that although BAT is present in the marsupial *S. crassicaudata*, it may not be important for thermogenesis, at least in the short term. *S. crassicaudata* utilises a plasticity in body temperature and a change in



substrate utilisation in order to maintain energy balance and body composition without the need for an increase in metabolic rate or food consumption and without the need for torpor, provided adequate food is available.

## 13.2 INTRODUCTION

When exposed to low environmental temperatures, mammals maintain their body temperature by a variety of mechanisms. These include behavioural modifications such as huddling, alterations in the insulative properties of skin by peripheral vasoconstriction, and shivering and non-shivering thermogenesis (NST) (Wallis 1982). NST is achieved primarily by the production of heat in brown adipose tissue (BAT) (see section 1.7.3). The role of BAT varies substantially between species. In some species, for example ruminants, BAT is retained for only a very brief period in post-natal life (Casteilla *et al* 1989), whereas in many hibernators and eutherian rodents, BAT is the principal source for heat generation throughout life (Trayhurn *et al* 1987).

BAT has a characteristic appearance on histological examination (multilocular, numerous electron dense mitochondria) and binds guanosine di-phosphate (GDP) within its mitochondria (Himms-Hagen 1985, Trayhurn & Milner 1989). However, histological characteristics and GDP binding are insufficient to prove the presence of BAT (Trayhurn *et al* 1987). For example, the biological features of white adipose tissue of cold exposed rats are similar to those normally associated with brown fat

(Loncar *et al* 1988a,b), and in *ob/ob* mice brown adipocytes appear unilocular (a characteristic of white adipocytes) due to abnormally high triacylglycerol stores (Trayhurn 1993). A 32 kd mitochondrial uncoupling protein (UCP1), specific to BAT, has been shown to regulate the production of heat through the uncoupling of oxidative phosphorylation (Henningfield & Swick 1987, Lean & James 1983, Trayhurn 1993). The detection of UCP1 has been used as a marker for the presence of BAT in a wide range of mammals, including non-hibernating rodents (rats, mice), hibernating mammals (ground squirrels and hamsters), carnivores (dogs), ruminants (lambs, cattle and goats), and primates (monkeys and humans) (Brander *et al* 1993, Henningfield & Swick 1987, Lean & James 1983, Milner & Trayhurn 1990, Trayhurn *et al* 1987).

Interestingly, UCP1 deficient mice are cold sensitive which suggests that UCP1 in BAT is important for thermoregulation (Enerback *et al* 1997). Furthermore, BAT has been implicated as being an important site for facultative energy expenditure, and the BAT knockout mouse is both obese and cold sensitive, implying a role for BAT not only in thermogenesis but also in energy balance (Lowell *et al* 1993). In contrast to the BAT knockout mouse, UCP1 knockout mice are not obese, but they do show an up-regulation of UCP2 (Enerback *et al* 1997). UCP2, a protein with high homology to UCP1, is widely expressed in mammalian tissues (Fleury *et al* 1997, Gimeno *et al* 1997) (see section 1.7.3.3). While UCP2 is thermogenic *in vitro*, it appears to be more important in the regulation of nutrient partitioning *in vivo*, via alterations in free fatty acid oxidation within the mitochondria (Fleury *et al* 1997, Simoneau *et al* 1998, Solanes *et al* 1997). Regardless of its mechanism of action, it would appear that

increased UCP2 expression prevents obesity but not cold sensitivity in the UCP1 knockout mouse. The physiological role of UCP2 has not yet been clearly elucidated.

The presence of BAT in marsupials is controversial (see section 2.3.2.1); electron microscopy and GDP binding studies in isolated mitochondria have suggested that BAT is present in young Bennett's Wallabies (*Macropus rufogriseus*) (Loudon *et al* 1985), while examination of potoroo (*Potorous tridactylus*) adipose tissue has failed to identify BAT based on histological characteristics (Nicol 1978). Attempts to identify UCP1 in marsupials have been unsuccessful (Trayhurn 1993). Furthermore, as far as can be determined, UCP2 has not previously been looked for in a marsupial.

*S. crassicaudata* has a thermoneutral zone from 31.5 to 35.5 °C, which is often higher than ambient temperatures of its natural environment (MacMillen & Nelson 1969). *S. crassicaudata* shows a variety of responses to cold including huddling, nest sharing, basking, reduced activity and torpor (Wallis 1982). Torpor occurs in the wild following dry and cold nights when food availability is limited, and is associated with a progressive decline in metabolic rate (Frey 1991). In the laboratory, *S. crassicaudata* enter torpor following fasting and cold exposure (Geiser & Baudinette 1987, Godfrey 1968). During torpor bouts, which can last from 50 minutes to over 18 hours, body temperature can drop to as low as 10 °C (Holloway & Geiser 1995).

The aim of this study was to determine in *S. crassicaudata*, i) whether BAT is present in interscapular fat depots, ii) the response to acute and chronic cold exposure, iii)

whether interscapular adipose tissue is used during re-warming from torpor, and iv) the presence of UCP 2 mRNA expression.

### 13.3 MATERIALS AND METHODS

#### 13.3.1 *Experimental animals and diet*

Adult male *S. crassicaudata* housed under a long-day light regime (16-h light, 8-h dark; lights turned on at 23:30h) were used in all studies. Animals were housed at 28 °C unless otherwise specified. Standard laboratory diet was provided *ad libitum* except where stated. Food intake was expressed as kcal/g body weight.

#### 13.3.2 *Experimental protocols*

##### Study 1: Effect of acute cold exposure

Eight adult male *S. crassicaudata*, initially housed at an environmental temperature of 28 °C, were exposed to an ambient temperature of 15 °C. Basal body temperature ( $T_b$ ) was initially measured at 28 °C, and then every 30 minutes for 150 minutes during cold exposure. Animals were then returned to 28 °C and  $T_b$  was recorded every 30 minutes for a further 120 minutes.

##### Study 2: Effect of prolonged cold exposure

‘Baseline’ oxygen consumption ( $VO_2$ ), respiratory quotient (RQ), tail width, body weight, 24-hour food intake and body temperature were determined in 4 *S. crassicaudata* maintained at an environmental temperature of 28 °C. Animals were

subsequently placed at 15 °C. VO<sub>2</sub> was measured on *days 6 and 12*, and body temperature was recorded on *days 2, 5, 6, 7, 10 and 12*. Food intake, body weight and tail width were measured daily. Animals were killed on *day 13* by rapid decapitation. Interscapular and tail adipose tissue was dissected out from these animals, as well as from a group of 4 control male *S. crassicaudata* housed at 28 °C. In addition, interscapular adipose tissue was taken from 2 adult mice housed at 28 °C for 48 hours, and 2 adult mice housed at 4 °C for 48 hours. Tissue was snap frozen in liquid nitrogen and stored at -80 °C until analysis.

*Study 3: Determination of the use of interscapular adipose tissue during re-warming from torpor*

Male *S. crassicaudata* ( $n = 28$ ) initially housed at an environmental temperature of 24 °C were used in this study. Torpor and re-warming were induced as stated in section 13.3.4. Animals were sacrificed at predetermined times by an intraperitoneal injection of sodium pentobarbitone (600 mg/kg) (Boehringer Mannheim, NSW, Australia). Animals killed prior to commencement of re-warming were termed ‘-15 min animals’. Time points commenced from the moment body temperature reached 30 °C, and were termed (0, 5, 10, 15, 30 and 60 minutes). Interscapular adipose tissue was dissected from ‘warm active’ animals, and animals at -15, 0, 5, 10, 15, 30 and 60 minutes after arousal from torpor ( $n = 4$  per group). Tissue was snap frozen in liquid nitrogen and stored at -80 °C until analysis.

### 13.3.3 *Metabolic parameters*

Oxygen consumption ( $\text{VO}_2$ ) and respiratory quotient (RQ) were determined at 28 °C for each animal using the methods outlined in section 3.3.4. 'Baseline'  $\text{VO}_2$  was defined as the most stable value reached at least 30 minutes after the introduction of the animal into the chamber.

### 13.3.4 *Induction of torpor and subsequent re-warming*

Torpor was induced using a standard protocol (Langman *et al* 1996, Orgeig *et al* 1996). Briefly, all food was removed from cages at 17:00h on the day prior to the experiment. The following day at 09:00h, initial body temperature was recorded to confirm a  $T_b$  of  $> 34^\circ\text{C}$ . In this condition *S. crassicaudata* are alert and so were termed 'warm-active'. The ambient temperature was then reduced to 10 °C. Torpid animals were identified by a characteristic body posture, which included either lying flat with splayed legs or curled tightly into a ball (Langman *et al* 1996). Once a torpid posture was adopted,  $T_b$  was again recorded to confirm torpor. Animals with a  $T_b$  lower than 20 °C were defined as torpid. Torpid animals were checked hourly to ascertain that they did not rouse from torpor during this time. *S. crassicaudata* were maintained in torpor for 8 hours before the commencement of re-warming.

Rewarming was achieved by holding the animal firmly within the gloved hand of the experimenter. This method of rewarming has been described previously (Langman *et al* 1996), and produces a rapid temperature rise of 1-2 °C/min. Body

temperature was recorded once during the arousal period and once again when the animals were warm and active.

### **13.3.5 Characterisation of adipose tissue**

Electron microscopy and biochemical analysis of interscapular adipose tissue was carried out as stated in sections 3.7.2 and 3.7.3.

### **13.3.6 Northern blotting**

#### **13.3.6.1 UCP1 and UCP2 cDNA probes**

A 27 base pair, DIG labelled, UCP1 oligonucleotide probe, as described in section 3.8.2.1, was used to detect UCP1 mRNA, whereas a 1.1 kb human UCP2 cDNA probe labelled with [ $\alpha$ - $^{32}$ P]dCTP to a specific radioactivity of  $1 \times 10^9$  dpm/ $\mu$ g was used to detect UCP2 mRNA (see section 3.8.2.2).

#### **13.3.6.2 RNA extraction, gel electrophoresis and Northern blotting**

Total RNA was isolated from *S. crassicaudata* white adipose tissue, interscapular adipose tissue, cortex, muscle, liver, bowel, spleen, kidney, and heart using the method outlined in section 3.8.1. RNA was also isolated from mouse white adipose tissue, interscapular brown adipose tissue, and cortex.

Agarose gel electrophoresis and was performed as described in section 3.9.1.

Northern blotting using the DIG labelled UCP1 oligonucleotide was performed as outlined in section 3.8.4. Blots were exposed for 30 minutes. Northern blotting using

the [ $\alpha$ - $^{32}$ P]dCTP labelled UCP2 cDNA probe was performed as described in section 3.8.3. The UCP2 probe was used at  $1.25 \times 10^6$  counts/ml, and blots were exposed for 5 days at  $-80^\circ\text{C}$ .

### 13.3.7 *Statistical analyses*

Statistical analyses were carried out as stated in section 3.9.

## 13.4 RESULTS

### 13.4.1 *Electron microscopic characterisation of adipose tissue*

*Interscapular adipose tissue:* The cytoplasm of the adipocytes contained several fat droplets (*ie.* was multilocular), and one of these was very large and occupied most of the cell volume pushing the nucleus and the cytoplasm into the periphery of the cell. The thin rim of cytoplasm at the periphery and around the nucleus contained a large number of electron dense mitochondria. Furthermore, the extracellular matrix between adipocytes had many blood vessels, Figs 13.1A, 13.2A.

*Tail adipose tissue:* Adipocytes were large and the cytoplasm contained a single large fat droplet that occupied most of the cell volume (*ie.* unilocular), pushing the nucleus and cytoplasm to the periphery of the cell. Compared to interscapular adipose tissue, the cytoplasm (of tail adipocytes) contained a very small number of electron dense mitochondria and the extracellular matrix between adipocytes had fewer blood vessels than interscapular adipose tissue, Fig 13.1B, 13.2B.



#### 13.4.2: *Study 1: Acute cold exposure*

After exposure to 15 °C for 30 minutes, body temperature decreased from a baseline of  $34.7 \pm 0.33$  °C to  $33.0 \pm 0.38$  °C ( $P < 0.001$ ) and reached a nadir of  $32.0 \pm 0.4$  °C at 90 minutes, after which it was maintained at this level. After return to an environmental temperature of 28 °C, body temperature increased to  $33.7 \pm 0.28$  °C within 30 minutes, ( $P < 0.001$ ) and by 120 minutes had stabilised at  $34.8 \pm 0.23$  °C, Fig 13.3.

#### 13.4.3 *Study 2: Effect of prolonged cold exposure*

##### 13.4.3.1 *Body temperature*

Following exposure to 15 °C, body temperature decreased from a baseline of  $35.1 \pm 0.01$  °C to  $34.45 \pm 0.23$  °C ( $P < 0.05$ ) after 24 hours. The lowest levels were recorded on *days 6* and *7* when temperatures were  $33.25 \pm 0.54$  °C and  $33.33 \pm 0.10$  °C respectively ( $P < 0.005$ ). Body temperatures remained low throughout the rest of the study, and was  $33.8 \pm 0.55$  °C on *day 12*. None of the animals were observed to enter torpor at any time, Fig 13.4A.

##### 13.4.3.2 *Oxygen consumption, CO<sub>2</sub> production, and respiratory quotient.*

*Day 0* oxygen consumption was  $11.62 \pm 0.15$  ml/g/h, and this decreased to  $9.79 \pm 0.38$  ml/g/h ( $P < 0.01$ ) on *day 6*, without a significant change thereafter, Table 13.1. There was no change in VCO<sub>2</sub> throughout the experiment. Respiratory quotient (RQ), increased from  $0.63 \pm 0.03$  at *day 0* to  $0.82 \pm 0.03$  by *day 6* ( $P < 0.001$ ), with no significant change after that time, Table 13.1.

#### 13.4.3.3 *Body weight, tail width and food intake.*

After 24 hours exposure to 15 °C, body weight increased ( $17.01 \pm 0.42$ g compared to baseline  $16.16 \pm 0.43$  g,  $P < 0.01$ ) and remained elevated until *day 2* ( $16.81 \pm 0.55$  g). A significant fall in body weight occurred on *day 3* ( $15.28 \pm 0.48$ g), after which body weight remained constant, and not significantly different from baseline, Fig 13.4B.

Mean tail width, which was initially  $4.3 \pm 0.4$  mm, decreased to  $4.0 \pm 0.4$  mm by *day 5* ( $P < 0.04$ ), then decreased further by *day 8* to  $3.7 \pm 0.4$  mm ( $P < 0.05$ ), after which it remained constant until the end of the study, Fig 13.4C.

There was no significant change in food intake during the course of the experiment, Fig 13.4D.

#### 13.4.3.4 *Biochemical analysis of interscapular adipose tissue*

At the end of the experiment the total mass of the interscapular fat was 66.6 mg and 60.0 mg in the control and cold exposed groups respectively. The percentage of total protein was 2.6% in the cold exposed and 2.1% in the control animals. Mitochondrial protein and DNA in interscapular fat was significantly greater in the cold exposed than the controls (500 vs 330  $\mu$ g/mg, and 1.8 vs 0.7  $\mu$ g/mg respectively), Table 13.2.

GDP binding in interscapular fat was lower in *S. crassicaudata* maintained at 15 °C (75 pm/mg protein) than in the control animals maintained at 28 °C (128 pm/mg protein), Table 13.2. This compares to 312 pm/mg protein and 509 pm/mg protein

from interscapular fat in mice housed for 48 hours at 28 °C and 4 °C respectively. GDP binding was not detected in tail fat deposits of *S. crassicaudata* housed at 28 °C.

In the animals maintained in an environment at 15 °C for 12 days, UCP1 immunoreactivity was clearly detected in the 32 000 Mr region by Western blot analysis, Fig 13.5. By contrast, UCP1 immunoreactivity was not detectable in animals maintained at 28 °C.

#### **13.4.4      *Study 3: Determination of the use of interscapular adipose tissue during re-warming from torpor***

There was no evidence that BAT metabolism was involved in the re-warming from torpor. Mitochondrial protein content was not significantly different between any of the time-points, and there was no significant change in GDP binding during arousal from torpor, Table 13.3. UCP1 immunoreactivity was not detectable in any of the samples .

#### **13.4.5      *Detection of UCP1 and UCP2 on a Northern blott***

##### ***13.4.5.1              UCP1***

Northern blot analysis revealed the expression of UCP1 mRNA as a single 2.2 kb band in both WAT and BAT of *S. crassicaudata* housed at 24 °C. UCP1 expression was also detected in BAT from a mouse, with bands detected at 1.5 kb and 1.2 kb. The probe did not detect UCP1 mRNA in mouse WAT, Fig 13.6.

#### 13.4.5.2 *UCP2*

Northern blot analysis revealed expression of UCP2 mRNA as a 1.8 kb band in all tissues of both mouse and *S. crassicaudata*. UCP2 mRNA was expressed at greater levels in *S. crassicaudata* spleen, liver, muscle, adipose tissue, and cortex, with lower levels of expression in kidney, heart and bowel, Fig 13.7.

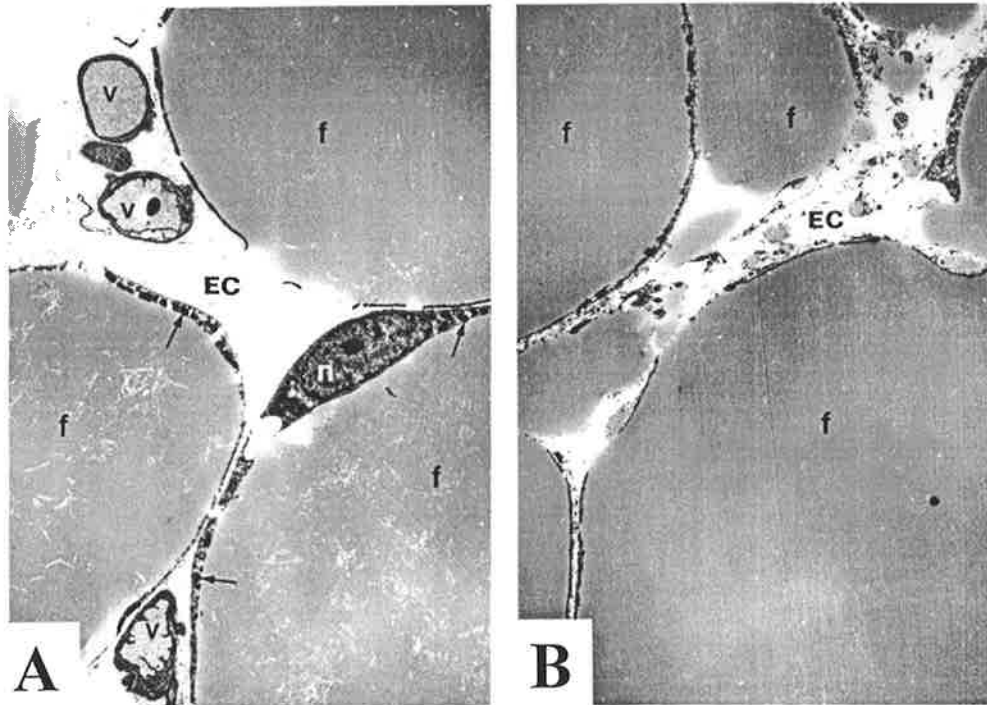


**Figure 13.1:** Electron micrographs of interscapular (A) and tail (B) adipose tissues from *S. crassicaudata*. In both tissues, large intracellular fat droplets (f) in the adipocytes have pushed the cytoplasm and nucleus (n) into a narrow rim at the periphery. In the cytoplasm of adipocytes from interscapular fat, there is a large number of electron-dense mitochondria (arrows). Note the presence of a large number of blood vessels (v) in the extracellular matrix (EC) of interscapular fat. Magnification: x 2,700.

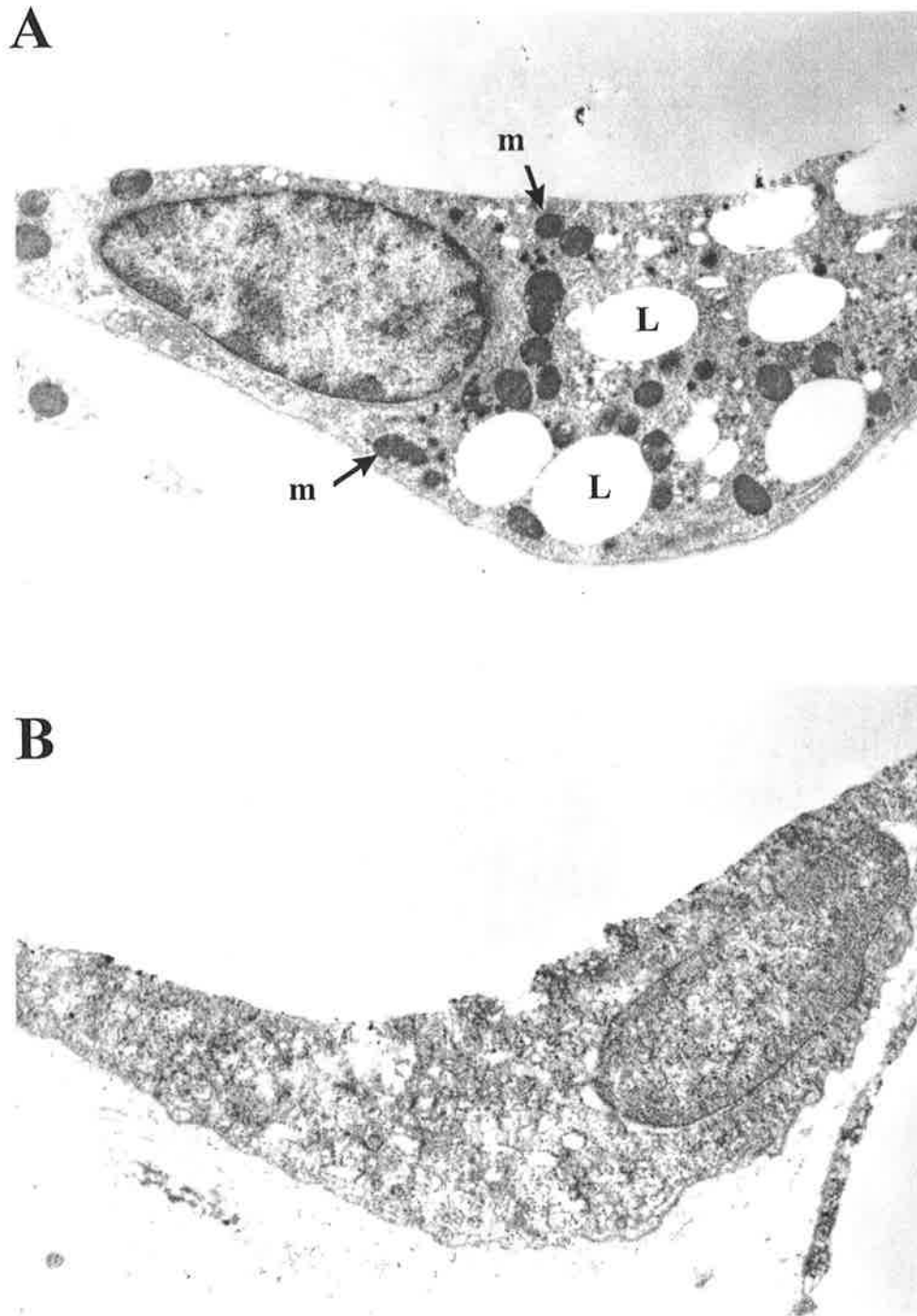


**Figure 13.2:** Electron micrographs of interscapular (A) and tail (B) adipocytes from *S. crassicaudata*. The adipocyte from the interscapular fat pad contains numerous electron dense mitochondria (m) within the cytoplasm and is multilocular (L). In contrast, the adipocyte from tail adipose tissue is void of mitochondria. Magnification: x 20,000.

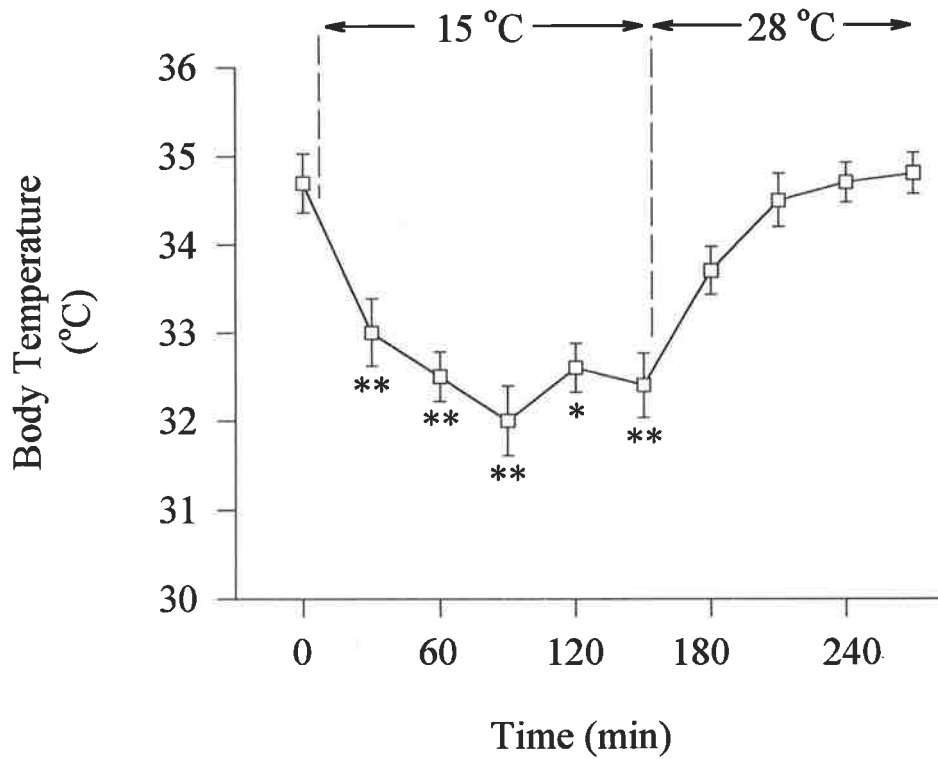


**Figure 13.3:** Acute changes in body temperature ( $T_b$ ) of male *S. crassicaudata* housed at 28 °C, when exposed to 15 °C for 150 minutes followed by a return to 28 °C.  $T_b$  fell ( $P < 0.001$ ) within 30 minutes of exposure to cold and rose to original levels ( $P < 0.001$ ) when animals were returned to 28 °C. Values are mean  $\pm$  SE;  $n = 8$ . \*  $P < 0.01$ ; \*\*  $P < 0.001$ .

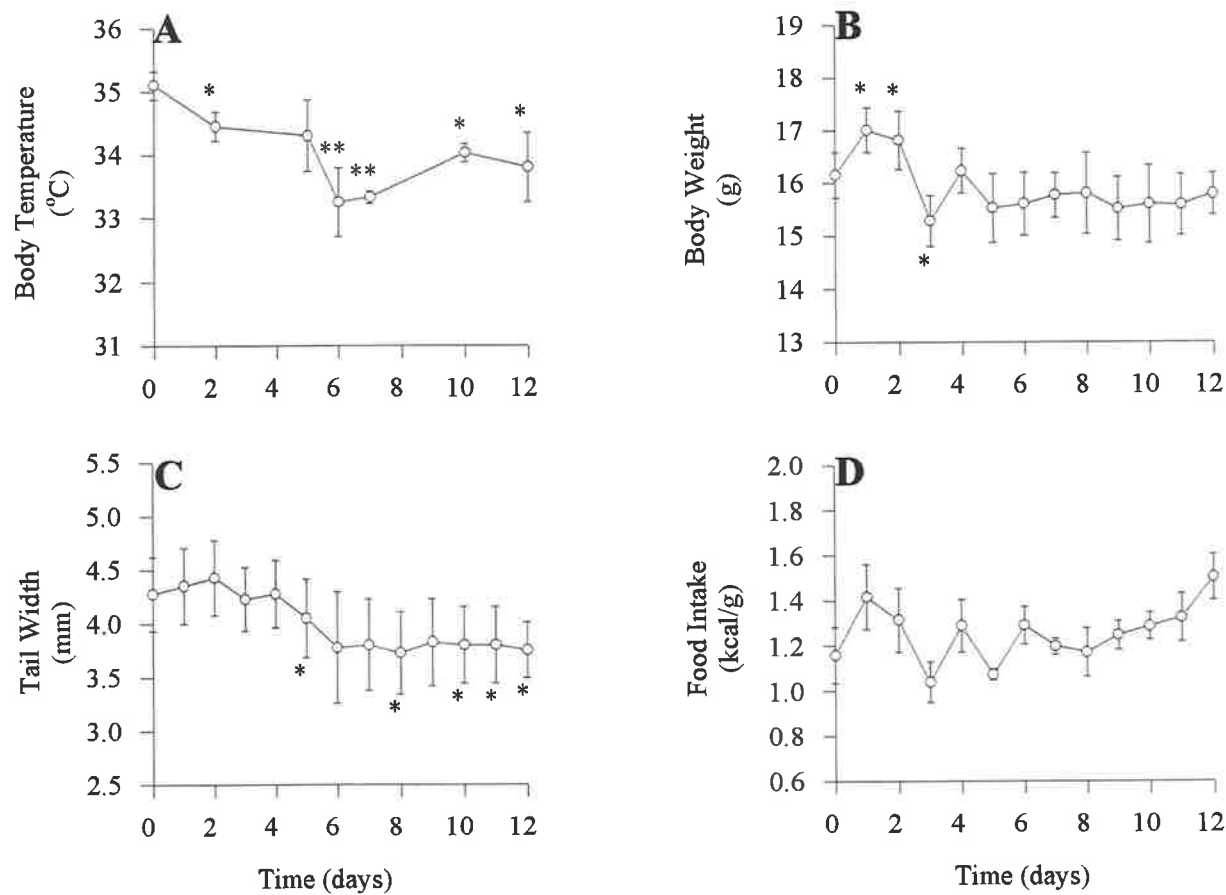


**Figure 13.4:** Effect of exposure to 15 °C for 12 days on body temperature (A; °C), body weight (B; g), tail width (C; mm), and 24-hour food intake (D; kcal/g body weight), in male *S. crassicaudata* originally housed at 28 °C. Body temperature showed a significant decrease ( $P < 0.05$ ) within 24 hours of exposure to 15 °C. Body weight initially increased and then decreased before stabilising on day 4 at levels not significantly different from basal. Tail width showed a significant reduction ( $P < 0.04$ ) by day 5. Food intake showed no significant change. Values are mean  $\pm$  SE;  $n = 4$ . \*  $P < 0.05$ ; \*\*  $P < 0.005$ .



| DAY | VO <sub>2</sub> (ml/g/h) | VCO <sub>2</sub> (ml/g/h) | RQ            |
|-----|--------------------------|---------------------------|---------------|
| 0   | 11.62 ± 0.15             | 7.26 ± 0.39               | 0.63 ± 0.03   |
| 6   | 9.79 ± 0.38*             | 8.01 ± 0.54               | 0.82 ± 0.03 † |
| 12  | 9.17 ± 0.61 *            | 8.23 ± 1.01               | 0.89 ± 0.05 † |

**Table 13.1:** Changes in metabolic parameters of *S. crassicaudata* after exposure to 15°C. O<sub>2</sub> consumption (VO<sub>2</sub>) showed a significant decrease ( $P < 0.01$ ) by day 6, but there was no change in CO<sub>2</sub> production (VCO<sub>2</sub>). Respiratory quotient (RQ) increased significantly ( $P < 0.001$ ) by day 6. Values are mean ± SE. \*  $P < 0.01$ , †  $P < 0.001$ .

|                                                | 28 °C housed | 15 °C housed |
|------------------------------------------------|--------------|--------------|
| BAT weight<br>(g per animal)                   | 66.6         | 60.0         |
| Total protein<br>(mg)                          | 1.42         | 1.55         |
| Mitochondrial protein<br>(µg/mg total protein) | 330          | 500          |
| Mitochondrial DNA<br>(µg/mg protein)           | 0.7          | 1.8          |
| GDP binding (pm/mg<br>protein)                 | 128          | 75           |

**Table 13.2:** Parameters of interscapular adipose tissue in cold-exposed vs control *S. crassicaudata*. Mitochondrial protein and DNA increased after cold exposure whereas GDP binding slightly decreased. BAT, brown adipose tissue.

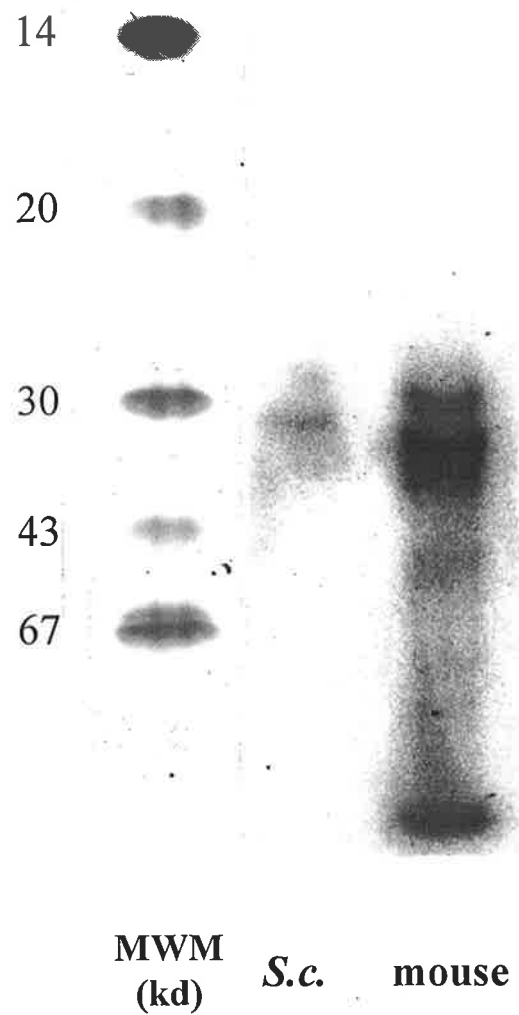


**Figure 13.5:** Western blot analysis showing uncoupling protein-1 (UCP1) immunoreactivity in 32-kDa region, in *S. crassicaudata* (*S. c.*) housed at 15 °C, and in mouse. MWM; molecular weight markers.

| Time Point<br>(min) | Mitochondrial<br>Protein<br>( $\mu\text{g}/\text{mg}$ tissue) | GDP Binding<br>( $\text{pmol}/\text{mg}$<br>tissue) | GDP Binding<br>( $\text{pmol}/\mu\text{g}$<br>mitochondrial<br>protein) |
|---------------------|---------------------------------------------------------------|-----------------------------------------------------|-------------------------------------------------------------------------|
| -15                 | 12.91                                                         | 31.4                                                | 2.43                                                                    |
| 0                   | 10.68                                                         | 24.2                                                | 2.27                                                                    |
| 5                   | 7.17                                                          | 19.8                                                | 2.76                                                                    |
| 10                  | 7.97                                                          | 17.6                                                | 2.21                                                                    |
| 15                  | 10.00                                                         | 39.6                                                | 3.96                                                                    |
| 30                  | 6.94                                                          | 30.8                                                | 4.44                                                                    |
| 60                  | 11.39                                                         | 33                                                  | 2.9                                                                     |
| warm-active         | 5.39                                                          | 19.8                                                | 3.67                                                                    |

**Table 13.3:** Parameters of interscapular adipose tissue deposits in warm-active *S. crassicaudata* and in animals immediately following arousal from torpor.

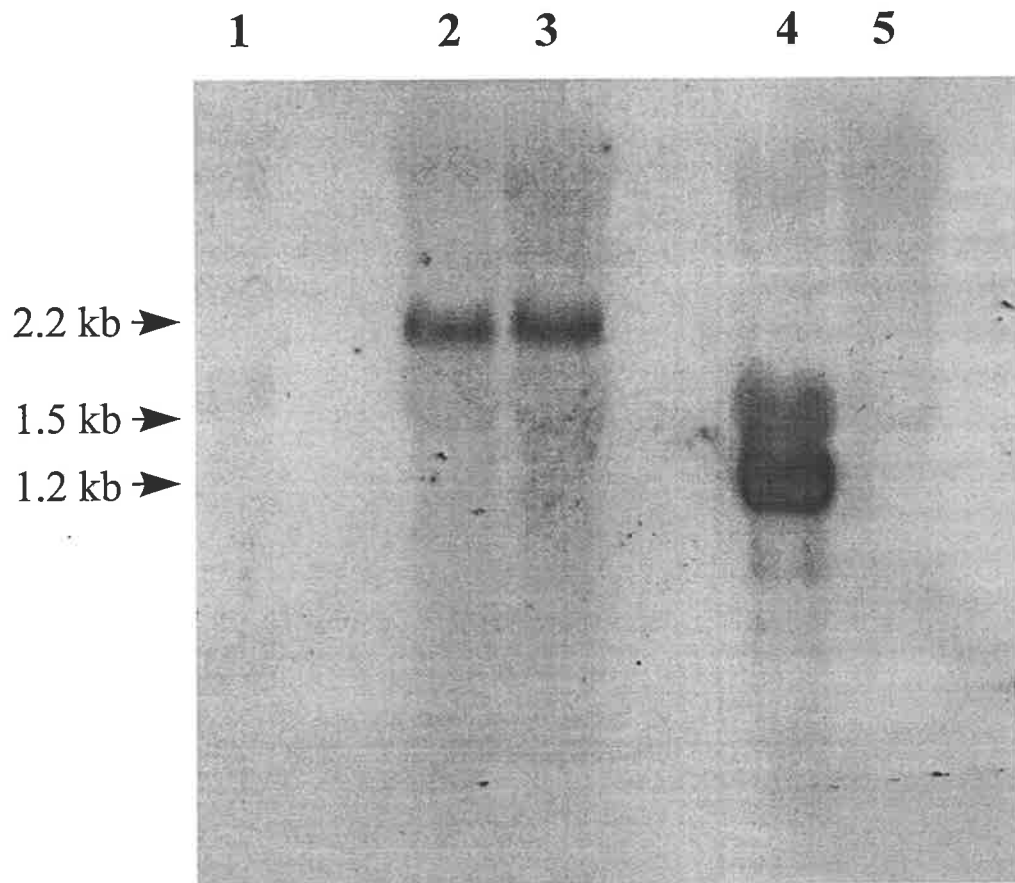


**Figure 13.6:** Northern blot of total RNA (20 µg) from *S. crassicaudata* WAT and BAT, and mouse WAT and BAT, probed with a DIG labelled UCP1 probe. Hybridisation signal was detectable in RNA from both WAT and BAT of *S. crassicaudata*, the transcript estimated to be ~ 2.2 kb. Hybridisation signal was detected at 1.2 kb in mouse BAT only. Lane 1 = molecular markers (Gibco BRL 0.24-9.5 kb, Life Technologies), Lane 2 = *S. crassicaudata* BAT, Lane 3 = *S. crassicaudata* WAT, Lane 4 = mouse BAT, Lane 5 = mouse WAT.

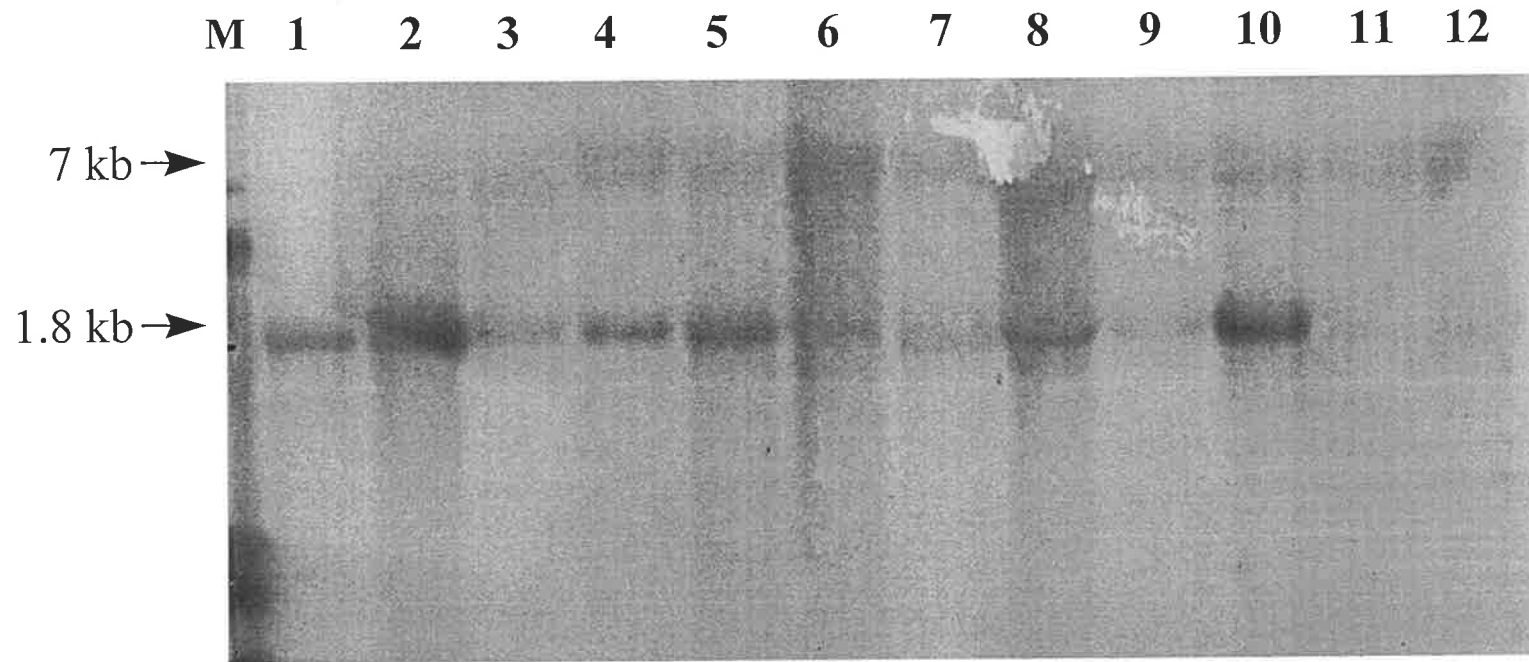


**Figure 13.7:** Northern blot of total RNA (20µg) from mouse WAT, BAT, and cortex, and from *S. crassicaudata* WAT, BAT, cortex, skeletal muscle, liver, bowel, spleen, kidney and heart. The blot was probed with a  $^{32}\text{P}$  labelled UCP2 probe. The estimated transcript size is 1.8 kb. Lane M = molecular markers (Gibco BRL 0.24-9.5 kb, Life Teechnologies), Lane 1 = mouse WAT, Lane 2 = mouse BAT, Lane 3 = mouse cortex, Lane 4 = *S. crassicaudata* WAT, Lane 5 = *S. crassicaudata* BAT, Lane 6 = *S. crassicaudata* cortex, Lane 7 = *S. crassicaudata* skeletal muscle, Lane 8 = *S. crassicaudata* liver, Lane 9 = *S. crassicaudata* bowel, Lane 10 = *S. crassicaudata* spleen, Lane 11 = *S. crassicaudata* kidney, Lane 12 = *S. crassicaudata* heart.

### 13.5 DISCUSSION

Using a combination of microscopic appearance, GDP binding and the detection of UCP1 protein and mRNA, the presence of BAT in interscapular fat of *S. crassicaudata* has been identified. None of the histological features of BAT were present in tail fat from *S. crassicaudata*, which exhibited characteristics typical of white fat deposits. The interscapular region is the primary location of BAT in the Siberian hamster (*Phodopus sungorus*) and many other rodent species (Himms-Hagen 1985, Wiesinger *et al* 1989). BAT has also been identified in many other groups of mammals including carnivores, primates, lagomorphs, ruminants and chiroptera (Trayhurn *et al* 1987). This is the first time that UCP1 has been detected in adipose tissue from a marsupial, and therefore the first unequivocal identification of BAT in this group of mammals.

UCP1 protein was not detectable in animals housed at 28 °C, but it was clearly identifiable in animals following 12 days of exposure to an ambient temperature of 15 °C. This finding is consistent with data from other species showing a rise in UCP1 following cold exposure (Trayhurn 1989, 1993). GDP binding was clearly present in the BAT from *S. crassicaudata*, but was considerably lower than in the BAT obtained from mice, and did not increase with cold exposure suggesting that BAT may not play an important role in maintaining basal body temperature in *S. crassicaudata*.

*S. crassicaudata* responded quickly to small fluctuations in environmental temperature by utilising a plasticity in body temperature, representing an important

mechanism for energy conservation. Several other small dasyurid marsupials such as *Sminthopsis froggatti*, *Dasyercus cristicaudata* and *Dasyurus geoffroii* show a similar ability to alter body temperature in response to changes in environmental temperatures, and they and also exhibit diurnal changes in body temperature. *S. crassicaudata* have similarly been shown to undergo diurnal variations in body temperature in the wild (Tyndale-Biscoe 1973). The field mouse (*Apodemus mystacinus*) initially decreases its body temperature when the environmental temperatures fall, but after 6 hours at 4 °C, body temperature returns to original levels (Haim *et al* 1993), presumably due to an increase in non-shivering thermogenesis (NST). Although the occurrence of NST in marsupials is controversial (see section 2.3.2.2), studies performed in this laboratory have shown that *S. crassicaudata* increase metabolic rate following noradrenaline administration, indicating NST (Clements *et al* 1998). The role, if any, that BAT plays in NST in *S. crassicaudata* is unclear.

The response of a reduced body temperature and total energy expenditure during prolonged cold exposure (*ie.* 15 °C for 12 days) appears to be an effective strategy to defend body composition. Although not measured directly, the decreased oxygen consumption of *S. crassicaudata* during cold exposure is likely to be the result of reduced activity. Decreased activity in response to cold is an energy conservation strategy previously demonstrated in wild *S. crassicaudata* (Wallis 1979, 1982). Despite the marked reduction in total energy expenditure, there was no decrease in food intake, and body mass (apart from an initial increase) remained stable. RQ values prior to cold exposure indicate that at 28 °C animals were primarily utilising fat for

energy. This observation is supported by the initial reduction in tail fat stores. The subsequent increase in RQ indicates a switch from fat to carbohydrate utilisation, presumably in order to preserve remaining fat stores. Our findings are in contrast to those in rats which show a fall in RQ which occurs within 60 minutes of cold exposure, and persists for its duration (Refinetti 1990). Rats, however, are homeothermic and maintain body temperature when exposed to cold by increasing metabolic rate and food intake (Harri *et al* 1984).

Prolonged cold exposure induced a decrease in GDP binding by approximately 50% as compared to animals housed at 28°C. These findings are in contrast to most other species (including mice) where both metabolic rate and GDP binding increase as environmental temperature falls (Trayhurn 1989). In hibernators such as the garden doormouse (*Eliomys quercinus*), high levels of GDP binding are observed during arousal from hibernation (Martins *et al* 1991). Arousal is associated with increased thermogenic activity of BAT through acute activation of pre-existing UCP1, by the unmasking of GDP binding sites (Martins *et al* 1991). In this study, GDP binding within the mitochondria of interscapular adipose tissue of *S. crassicaudata* did not significantly change during arousal from torpor. This suggests that increased heat generation from interscapular BAT of *S. crassicaudata* is not a major contributor to re-warming. As observed in other studies, the apparent lack of BAT in adult marsupials does not appear to slow their rate of re-warming from torpor (Lyman & O'Brien 1948), and the direct contribution of BAT to heat production during re-warming from torpor in other mammals could potentially have been over-estimated during the past (Geiser & Baudinette 1990).



It is likely that if food had not been available *ad libitum* to animals during this study they would have entered torpor. In the wild, *S. crassicaudata* must spend time foraging for the small insects that make up its diet as it cannot store food. *S. crassicaudata* does not readily go into torpor in the wild (Morton 1978d, Tyndale-Biscoe 1973). However, when temperatures fall and invertebrate numbers are low, *S. crassicaudata* will enter torpor, characterised by a decrease in body temperature and metabolic rate (Frey 1991, Morton 1978d). Torpor thus appears to be reserved as an emergency measure. The use of torpor for energy conservation has some disadvantages, including the increased chance of predation (Morton 1978b,d). Furthermore, although going into torpor can reduce energy requirements substantially, the daily energy savings during torpor are decreased by the energy costs of arousal, and it has been calculated that in order to save energy during torpor, a bout must last for at least 1 hour (Frey 1991).

In this study, oxygen consumption values obtained were higher than those which have previously been reported for *S. crassicaudata* (Geiser & Baudinette 1987, Holloway & Geiser 1995). In previous studies, oxygen consumption was measured over longer periods of time (up to 23 hours), during which animals were deprived of food and water. Consequently, these previous measurements are more representative of basal metabolic rates (see section 1.7.1). In the current study, oxygen consumption was used as an indicator of total energy expenditure, and it was measured within 30 minutes of the animal being placed into the metabolic chamber.

*S. crassicaudata* expresses UCP2 mRNA in a similar wide tissue distribution to other mammals (Fleury *et al* 1997, Gimeno *et al* 1997), however the role that UCP2 plays in energy balance in marsupials is unknown. There is some data to suggest that the expression of UCP2 in *S. crassicaudata* is affected by gonadal steroids (see chapter 14) and leptin (G Wittert, personal communication). In addition, recent studies in this laboratory have shown that UCP2 mRNA is expressed in the tammar wallaby (*Macropus eugenii*). UCP2 expression and oxygen consumption are higher in 200 as compared to 150 day old young wallabies, suggesting that UCP2 may have a thermogenic role under certain circumstances (G Wittert & R Baudinette, personal communication). In contrast, most studies performed in humans have found no correlation between UCP2 content and basal metabolic rate (Simoneau *et al* 1998). The physiological role of UCP2 therefore remains unclear.

*S. crassicaudata* demonstrates a range of alternative mechanisms for the maintenance of energy balance and body composition in response to changing environmental temperatures, rather than a dependence on either torpor or BAT. Further studies are needed to determine the specific role that BAT plays in regulating energy balance in marsupials. UCP2 may be more important to marsupials for the regulation of energy balance, via alterations in nutrient partitioning, although this has yet to be confirmed.

## CHAPTER 14

**THE INTERACTION OF OVARIAN STEROIDS AND PHOTOPERIOD AFFECTS BODY FAT STORES IN *S. CRASSICAUDATA*.****14.1 SUMMARY**

In many mammalian species, adipose tissue mass fluctuates in response to photoperiod and/or changes in the production of gonadal steroids. The relationships between changes in photoperiod and alterations in gonadal steroids, and the subsequent effects on body composition, however, are complex and differ between species. To determine the effects of photoperiod and ovarian steroids on fat stores in the marsupial *S. crassicaudata*, animals were ovariectomised (OVX) or sham operated, and maintained under either short-day (SD; 8-h light, 16-h dark) or long-day (LD; 16-h light, 8-h dark) photoperiods for 104 days. Photoperiod had no effect on body weight in the sham animals. In the LD OVX animals, body weight decreased and remained below baseline for about 45 days ( $P < 0.02$ ); after this time it returned to baseline. In contrast, there was an increase in body weight of SD OVX animals over the first 45 days ( $P < 0.0001$ ) with a subsequent return to baseline. Tail width (a reflection of body fat stores) increased in both sham and OVX animals exposed to SD ( $P < 0.05$ ). When exposed to LD, tail width increased only in the OVX animals ( $P <$

0.01). There was no effect of either photoperiod or OVX on total cumulative energy intake. Leptin mRNA expression was greater in the LD OVX as compared to the LD sham animals ( $P = 0.04$ ), and tended to be higher in SD when compared to LD shams ( $P = 0.09$ ). Photoperiod had no effect on UCP2 mRNA expression in either muscle or white adipose tissue (WAT) of the LD or SD shams. In skeletal muscle, UCP2 expression was lower in LD OVX when compared to LD shams ( $P = 0.02$ ). SD OVX animals had lower UCP2 expression in WAT when compared to LD OVX animals ( $P = 0.04$ ). These data indicate that in *S. crassicaudata*: (i) both SD photoperiod and OVX have a synergistic effect to increase fat mass, (ii) the effects of photoperiod on fat mass are mediated by both gonadal steroid-dependent and independent mechanisms, (iii) alterations in UCP2 mRNA expression may mediate the effect of OVX, but not photoperiod, and (iv) UCP2 mRNA is regulated differentially in muscle and fat.

## 14.2 INTRODUCTION

In many mammals, seasonal changes in photoperiod affect physiological functions including reproduction (Beasley & Zucker 1986, Sullivan & Lynch 1986), moulting (Sullivan & Lynch 1986), thermoregulation (Heldmaier *et al* 1989), and activity of brown adipose tissue (BAT) (Heldmaier *et al* 1981, McElroy *et al* 1986, McElroy & Wade 1986, 1987, Wade 1983). Numerous mammalian species also exhibit photoperiod dependent fluctuations in body weight and adipose tissue mass, reflecting changes in energy balance (Wade *et al* 1985, Wade & Bartness 1984a,b). In some

animals, changes in photoperiod may influence production of gonadal steroids, which may also affect adiposity (Dark *et al* 1984). Fluctuations in circulating gonadal hormone levels have been shown to influence body composition in a number of mammalian species. For example, ovariectomy increases adiposity in Syrian hamsters (*Mesocricetus auratus*), meadow voles (*Microtus pennsylvanicus*), guinea pigs, and rats (Bhatia & Wade 1989, Dark *et al* 1984, Richard 1986, Slob *et al* 1973). Conversely, oestrogen treatment is associated with reductions in both body weight and food intake in female rats, and a decrease in body weight of guinea pigs (Slob *et al* 1973, Wade *et al* 1985).

The relationships between changes in photoperiod and alterations in gonadal steroids and the subsequent effects on body composition are complex and differ between species. In meadow voles, ovariectomy increases body weight in long- but not short-day housed animals (Dark *et al* 1984). In contrast, in the pallid bat (*Antrozous pallidus*), gonadal hormones are not necessary for expression of annual body weight cycles; gonadal hormones may however influence the amplitude of these changes (Beasley & Zucker 1986). Therefore, although some species exhibit gonadal-dependent changes in adiposity in response to different photoperiods (Dark *et al* 1984), in others the response to photoperiod appears to be independent of gonadal status (Beasley & Zucker 1986).

Alterations in metabolic efficiency, rather than energy intake, may be responsible for the observed effects of photoperiod and ovarian steroids on adiposity in some species (Jones *et al* 1991). In Syrian hamsters, weight gain following exposure to short-days

or ovariectomy is not associated with alterations in food intake (Jones *et al* 1991, Wade & Bartness 1984b), whereas in rats weight gain following ovariectomy is accompanied by an increase in energy intake (Wade *et al* 1985). In both female meadow voles and Siberian hamsters (*Phodopus sungorus sungorus*) there is a decrease in body weight with short-day exposure (Dark *et al* 1984, Wade & Bartness 1984a). In contrast to the meadow vole, however, Siberian hamsters decrease their energy intake (Wade & Bartness 1984a). In Syrian hamsters, oestradiol-induced changes in energy storage are not associated with alterations in oxygen consumption or BAT thermic activity (Schneider *et al* 1986). In Siberian hamsters, decreased body weight with short photoperiods is accompanied by an increase in BAT mass, protein content and guanosine-di-phosphate (GDP) binding (Heldmaier *et al* 1981, McElroy *et al* 1986). Thermogenic activity in BAT is mediated by uncoupling protein-1 (UCP1) (Nicholls & Locke 1984). Recently, other uncoupling proteins, including UCP2 and UCP3, have been cloned (Boss *et al* 1997, Fleury *et al* 1997). Although UCP2 is not important for thermogenesis *in vivo*, there is considerable evidence that UCP2 may be important for metabolic efficiency, for example by modulating nutrient partitioning (Dulloo *et al* 1998, Samec *et al* 1998).

No studies to date have examined the role of photoperiod or gonadal steroids on fat storage in marsupials directly. Studies described in chapter 4 indicate that female *S. crassicaudata* reduce energy intake after five weeks exposure to a short-day photoperiod, and that this is not associated with alterations in fat mass. Furthermore, although *S. crassicaudata* expresses UCP1 in interscapular adipose tissue deposits, BAT does not appear to play a significant role in the regulation of energy balance (see

chapter 13). *S. crassicaudata* does, however, express UCP2 in a similar tissue distribution to humans and rodents (see chapter 13), but nothing is known about its regulation. Therefore, the aims of this study were to determine the effects of ovariectomy and photoperiod on adiposity, energy intake and UCP2 mRNA expression in *S. crassicaudata*.

### 14.3 MATERIALS AND METHODS

#### 14.3.1 *Experimental animals and diet*

Sexually mature female *S. crassicaudata* (aged 6-9 months) were used in this study. Animals were housed individually at  $24 \pm 1$  °C in a constant temperature cabinet (Quantec Precision Engineering, SA, Australia), initially under a long-day photoperiod (LD; 16-h light, 8-h dark). During the experiment, half of the animals in each treatment group were placed under a short-day light regime (SD; 8-h light, 16-h dark) while the other half remained under a long-day light regime. Animals were fed *ad libitum* on a standard laboratory diet. Data for food intake are expressed as kcal/g body weight.

#### 14.3.2 *Experimental protocols*

Forty long-day housed *S. crassicaudata* were divided into two groups of similar body weight and 24-hour food intake. One group was ovariectomised (OVX) ( $n = 20$ ); in the other group, sham surgery was performed ( $n = 20$ ). Ovariectomy and sham surgery were performed as described in sections 3.6 and 3.6.2. Following a recovery

period of 2-4 weeks, half of the animals in each group were exposed to short-day photoperiods (SD) ( $n = 10$ ), whereas the other half was maintained under long-day photoperiods (LD) ( $n = 10$ ). Animals were housed on these photoperiods for a period of 104 days. Food intake (see section 3.3.2) and body weight were measured at approximately the same time each day, tail width (see section 3.3.1) was measured every 2-3 days, and body temperature (see section 3.3.3) every week. Animals were sacrificed by rapid decapitation on day 104, and trunk blood taken for measurement of blood glucose (see section 3.4.1). Skeletal muscle and all visible sub-cutaneous adipose tissue was removed and snap frozen in liquid nitrogen before storage at  $-70^{\circ}\text{C}$ .

#### **14.3.3      *RNA extractions, agarose gel electrophoresis and Northern blotting***

Total RNA was extracted from sub-cutaneous white adipose tissue (WAT) and skeletal muscle by the method outlined in section 3.8.1. Total RNA (15-25  $\mu\text{g}/\text{lane}$ ) was electrophoresed and transferred as outlined in section 3.8.1.

A 1.8 kb cDNA probe for *S. crassicaudata* leptin (as described in section 3.8.2.3 and in chapter 6) was used. The full-length cDNAs were used to hybridise the RNA blots, and cDNA probes were labelled with  $[\alpha\text{-}^{32}\text{P}]$  dCTP to a specific radioactivity of approximately  $1 \times 10^9$  dpm/ $\mu\text{g}$  DNA according to the protocol outlined in section 3.8.2.3. Northern blotting was carried out as outlined in section 3.8.3.

Quantitation of gene expression following Northern blotting was carried out as outlined in section 3.8.5.



#### 14.3.4 *Statistical analyses*

Statistical analyses were carried out as stated in section 3.9.

### 14.4 RESULTS

#### 14.4.1 *Body weight*

At commencement of exposure to LD or SD photoperiods (*day 0*) body weights were  $16.17 \pm 0.52$  g,  $17.05 \pm 0.98$  g,  $17.29 \pm 1.09$  g and  $16.33 \pm 0.84$  g in the LD sham, SD sham, LD OVX, and SD OVX groups respectively; without any significant differences between the groups.

There was no overall effect of photoperiod on body weight in the sham-operated animals, Fig. 14.1A. There was a significant effect of photoperiod on body weight in the OVX animals (treatment  $\times$  day  $F_{82, 995} = 2.3$ ,  $P < 0.0001$ ), Fig 14.1B; the body weight of animals exposed to LD decreased, and remained below baseline for approximately 45 days, before returning to baseline by day 47, Fig 14.1B,C. By contrast, in SD OVX animals body weight increased above baseline over the first 45 days before returning to baseline by day 65, Fig 1B,D.

A significant effect of OVX on body weight was only seen in LD animals, where body weight decreased as compared to shams, however, this difference was only apparent for the first 45 days (treatment  $\times$  day  $F_{82,995} = 1.40$ ,  $P = 0.01$ ), Fig 14.1C. In contrast,

there was no difference in body weight of SD animals between sham and OVX groups, Fig 14.1D.

#### 14.4.2 *Tail width*

At the time of initial exposure to LD or SD photoperiods (*day 0*), tail widths were  $4.54 \pm 0.23$  mm,  $5.10 \pm 0.46$  mm,  $5.10 \pm 0.35$  mm and  $4.61 \pm 0.31$  mm in the LD sham, SD sham, LD OVX and SD OVX groups respectively, without any significant differences between the groups.

In the sham animals, there was a reduction in tail width in LD when compared to SD exposure (treatment  $F_{1,503} = 9.77$ ,  $P = 0.01$ , treatment  $\times$  day  $F_{41,503} = 3.77$ ,  $P < 0.0001$ ), Fig 14.2A. In SD sham controls, there was a small, but non-significant increase in tail width. In OVX animals, tail width increased following both LD and SD exposure, although there was no overall difference between these groups, Fig 14.2B. Over the first 23 days of the experiment, however, tail width increased more in LD when compared to SD exposed OVX animals (treatment  $F_{1,131} = 4.70$ ,  $P = 0.05$ , treatment  $\times$  day  $F_{10,131} = 3.52$ ,  $P = 0.0005$ ); in LD exposed animals, tail width decreased slightly thereafter, but with SD exposure tail width continued to gradually increase, Fig 14.2B.

Following LD exposure, OVX prevented the decrease in tail width evident with LD shams (treatment  $F_{1,503} = 11.62$ ,  $P = 0.007$ , treatment  $\times$  day  $F_{41,503} = 3.77$ ,  $P < 0.0001$ ), Fig 14.2C. With SD exposure, OVX induced a larger increase in tail width compared to that observed in the shams (although this difference was only significant

over the first 33 days of the experiment) (treatment  $\times$  day  $F_{14,179} = 2.07$ ,  $P = 0.02$ ), Fig 14.2D.

#### 14.4.3 *Energy intake*

Cumulative energy intake was  $65.24 \pm 4.15$  and  $65.9 \pm 3.97$  kcal/g of body weight in the LD and SD sham groups respectively, and  $63.04 \pm 3.74$  and  $59.58 \pm 3.71$  kcal/g of body weight in the LD and SD OVX groups respectively. There was no significant effect of either photoperiod or OVX on overall energy intake.

#### 14.4.4 *Body temperature*

At the time of initial exposure to LD or SD photoperiods (*day 0*), body temperature was  $34.19 \pm 0.29$  °C,  $34.62 \pm 0.27$  °C,  $34.34 \pm 0.31$  °C and  $34.30 \pm 0.30$  °C in the LD sham, SD sham, LD OVX and SD OVX groups respectively, and there were no significant differences between them. Neither photoperiod nor OVX had a significant effect on body temperature.

#### 14.4.5 *Leptin mRNA expression in white adipose tissue*

Leptin mRNA expression in WAT tended to be higher in SD as compared to LD sham animals ( $P = 0.09$ ), Fig 14.3A. In LD OVX animals leptin mRNA expression was higher when compared to the LD shams ( $P = 0.04$ ); leptin mRNA expression also tended to be higher in the SD OVX animals as compared to the SD shams ( $P = 0.08$ ), Fig 14.3A. There was a significant correlation between leptin mRNA expression and tail width ( $r = 0.533$ ,  $P = 0.009$ ), Fig 14.3B.

**14.4.6      *UCP2 mRNA expression in white adipose tissue and skeletal muscle***

In the sham animals, there was no effect of photoperiod on UCP2 mRNA expression in either WAT or skeletal muscle, Fig 14.4A,B. UCP2 expression in WAT was lower in SD OVX when compared to LD OVX animals ( $P = 0.04$ ), Fig 14.4A. UCP2 mRNA expression in skeletal muscle was lower in LD OVX animals compared to LD shams ( $P = 0.02$ ) and also lower than in the SD OVX animals ( $P = 0.02$ ), Fig 14.4B.

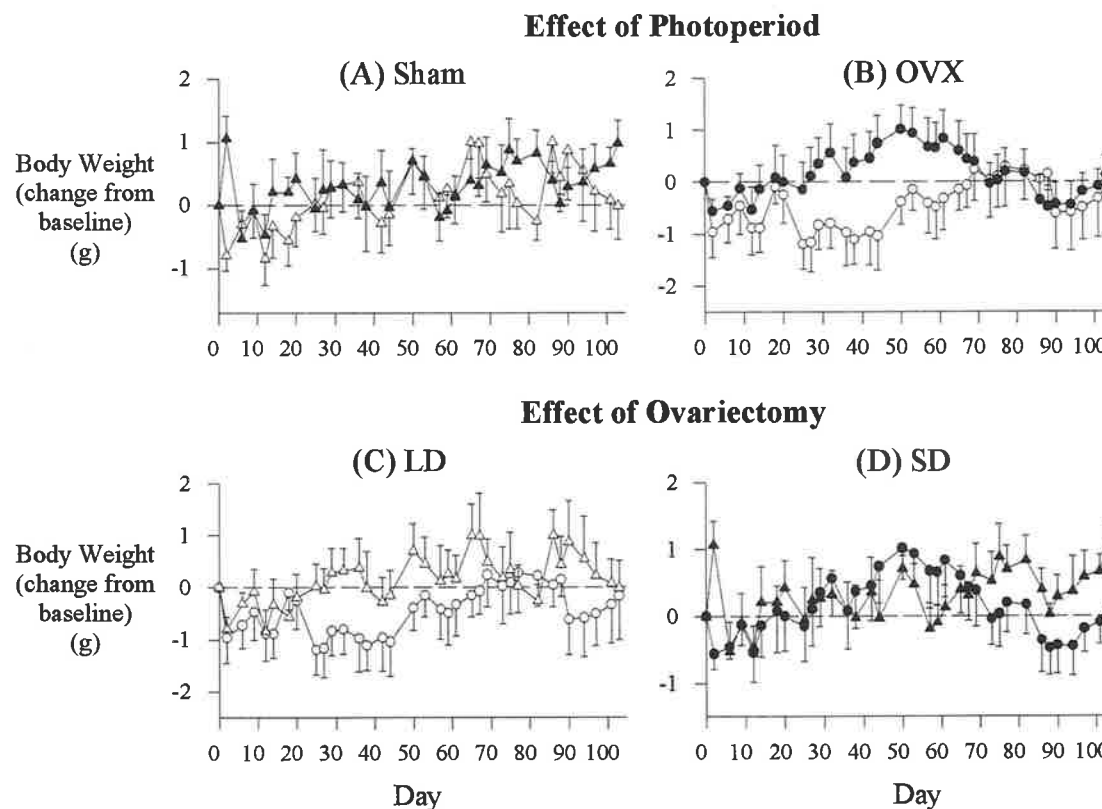


**Figure 14.1:** The effect of photoperiod on body weight in female *S. crassicaudata* that (A) underwent sham surgery and (B) were ovariectomised (OVX), and the effect of ovariectomy on body weight in (C) long-day housed (LD) and (D) short day housed (SD) animals. Body weight is expressed as change from baseline, and data are shown as mean  $\pm$  SEM. LD sham  $n = 7$ , SD sham  $n = 5$ , LD OVX  $n = 5$ , SD OVX  $n = 7$ . (Open triangle = LD sham, closed triangle = SD sham, open circle = LD OVX, closed circle = SD OVX).

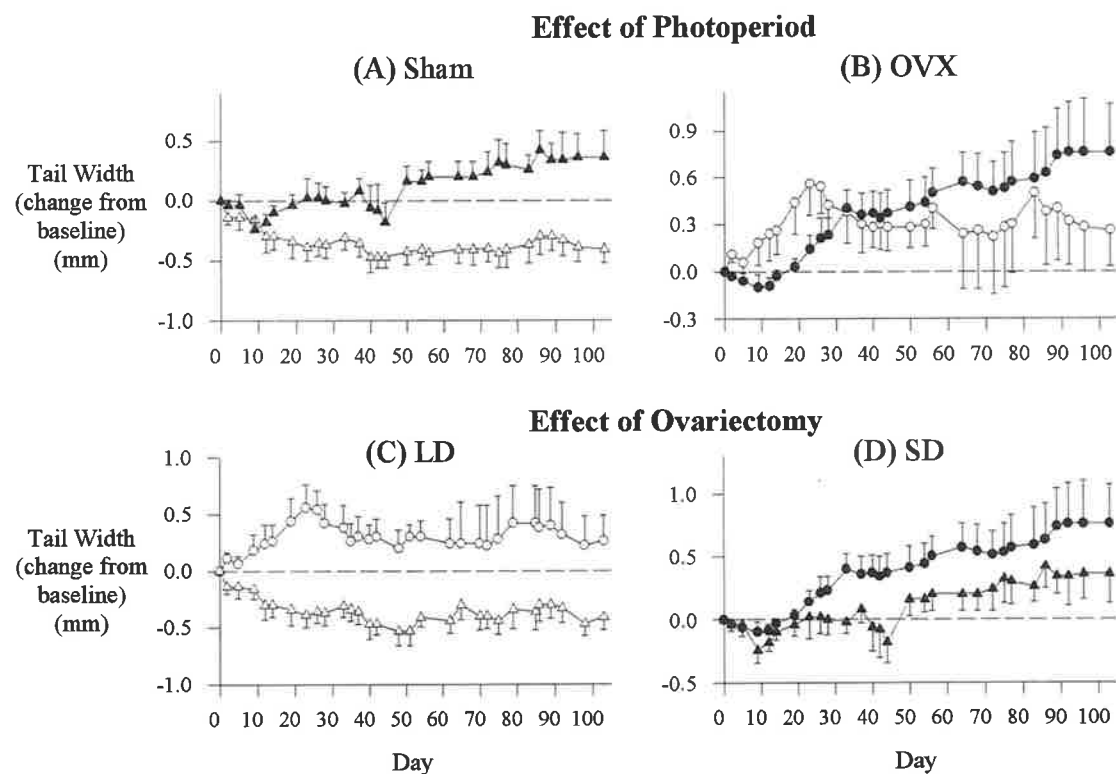


**Figure 14.2:** The effect of photoperiod on tail width in female *S. crassicaudata* that (A) underwent sham surgery and (B) were ovariectomised (OVX), and the effect of ovariectomy on tail width in (C) long-day housed (LD) and (D) short-day housed (SD) animals. Tail width is expressed as change from baseline, and data are shown as mean  $\pm$  SEM. LD sham  $n = 7$ , SD sham  $n = 5$ , LD OVX  $n = 5$ , SD OVX  $n = 7$ . (Open triangle = LD sham, closed triangle = SD sham, open circle = LD OVX, closed circle = SD OVX)

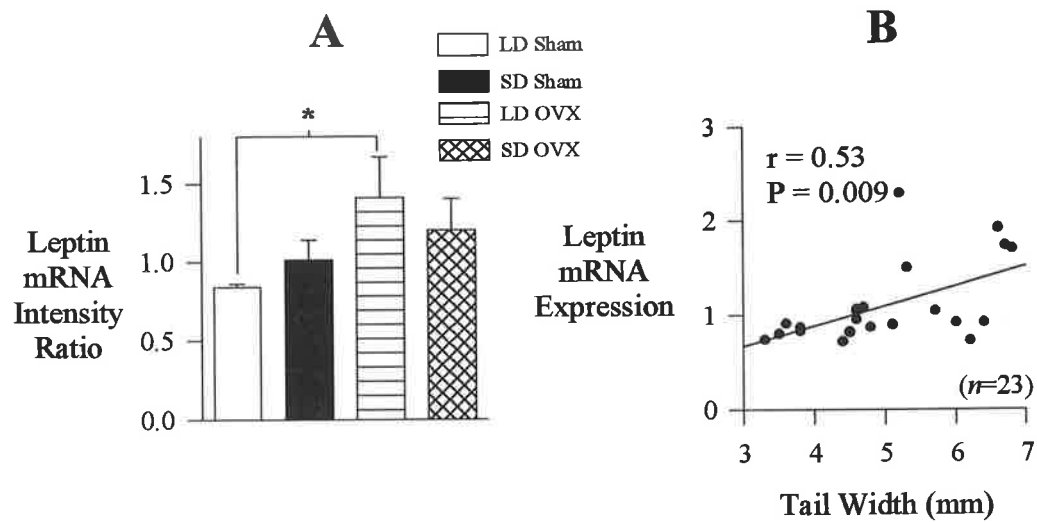


**Figure 14.3:** (A) Leptin mRNA expression (Mean  $\pm$  SE) in response to photoperiod and ovariectomy (OVX) in female *S. crassicaudata*. Both short-day photoperiod and OVX increased leptin mRNA expression. (B) correlation between tail width (a reflection of adipose tissue stores) and leptin mRNA expression in female *S. crassicaudata*.  $n = 23$ ; \*  $P < 0.05$ .

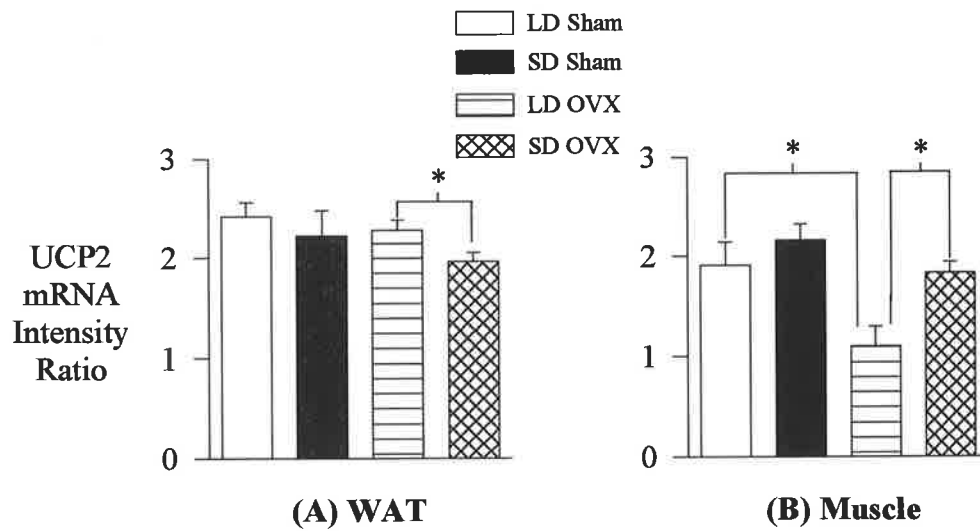


**Figure 14.4:** UCP2 mRNA expression in (A) subcutaneous white adipose tissue (WAT) and (B) skeletal muscle, in response to photoperiod and OVX in *S. crassicaudata*. In OVX animals exposed to SD UCP2 mRNA expression was reduced in WAT and increased in muscle, when compared to animals exposed to LD. OVX animals exposed to LD had lower UCP2 expression in muscle than LD shams. Values are Mean  $\pm$  SE; \*  $P < 0.05$ .



## 14.5 DISCUSSION

These studies establish that alterations in photoperiod and ovarian steroids affect fat mass in the marsupial *S. crassicaudata*, and their effects may be synergistic. Fat mass decreased in female *S. crassicaudata* housed under long-day (LD) as compared to short-day (SD) photoperiods reflecting a decrease and increase in fat mass of the LD and SD housed animals respectively. Ovariectomy (OVX) increased fat mass and decreased UCP2 mRNA expression in skeletal muscle, but these effects were modified by photoperiod; adiposity was greater in SD OVX than SD sham operated animals. UCP2 mRNA was marginally decreased in muscle of both LD and SD OVX animals, and only in adipose tissue in SD OVX animals.

Tail width is an indicator of body fat stores, reflecting the energy balance of *S. crassicaudata* in the wild and in the laboratory. In female animals, tail fat accounts for approximately 25% of total adipose tissue stores (see chapter 5); there are significant relationships between tail width and tail fat content, and between tail width and body fat content. This study has shown a significant correlation between tail width and leptin mRNA expression in sub-cutaneous white adipose tissue (WAT). In rodents and humans, the expression of leptin mRNA in WAT reflects the amount of fat (Frederick *et al* 1995a, Lonnqvist *et al* 1997, Maffei *et al* 1995).

Because tail fat in *S. crassicaudata* accounts for only a small percentage of total body weight (~3.5%), changes in tail width may not be reflected by changes in overall body weight and tail width is probably a more sensitive indicator of alteration in fat stores.

In this study, there was some discordance between the changes in tail width and changes in body weight. For example, body weight increased over the first 45 days in the SD OVX animals and decreased thereafter, whereas tail width continued to increase. By contrast, in the LD OVX animals body weight initially decreased despite an increase in tail width, suggesting a decrease in lean body mass.

In both female Syrian and Siberian hamsters changes in body weight in response to photoperiod or alterations in ovarian steroids are primarily due to changes in carcass lipid rather than lean body mass (Wade 1983, Wade & Bartness 1984a), but in these studies the carcass analysis was done at a single time point, at the end of the study. While a dynamic analysis of body composition, using for example dual x-ray absorptiometry, would clarify the changes observed in body composition, this was not feasible in the current study. Nevertheless, it appears that complex and dynamic changes in composition result from alterations in both photoperiod and gonadal steroids. The exact nature and mechanisms of each of these changes require clarification.

The observation that adiposity increases with SD exposure in *S. crassicaudata* is consistent with studies of *S. crassicaudata* in the wild (Morton 1978c), and in other mammals, such as the pallid bat and Syrian hamster (Beasley & Zucker 1986, Wade & Bartness 1984b). Although adiposity of female *S. crassicaudata* increased (as reflected by an increase in tail width and leptin mRNA expression in sub-cutaneous WAT), with SD exposure, overall energy intake did not alter, suggesting an increased metabolic efficiency or decreased energy expenditure. These findings are in contrast

to the observations in chapter 5 demonstrating a reduction in food intake of females following 5 weeks under a SD photoperiod. The reason for this discrepant response is unclear, however in both situations there appears to be a conservation of energy in SD females. SD photoperiod is associated with a reduction in energy expenditure in some rodents. For example, in the Syrian hamster a reduction in energy expenditure facilitates storage of additional carcass fat without placing extra demands on food supply, possibly by modifying voluntary activity and/or thermogenesis (Wade & Bartness 1984b). Spontaneous physical activity was not measured in this study, however, Holloway and Geiser (1996) have shown that under SD photoperiods, male *S. crassicaudata* decrease their physical activity and that this is not associated with changes in either basal metabolic rate or food intake (Holloway & Geiser 1996). Studies performed in this laboratory, have been unable to replicate the finding that SD males are less active than LD males (H Turnbull, personal communication); the reason for this discrepancy is not known.. No study to date has examined alterations in physical activity of female *S. crassicaudata* in response to photoperiod.

UCP2 mRNA expression was not affected by photoperiod in either sub-cutaneous WAT or skeletal muscle in the sham operated animals. The mechanisms regulating UCP2 are unclear, however UCP2 may be a key gene in the regulation of metabolic efficiency by altering nutrient partitioning (Dulloo *et al* 1998, Samec *et al* 1998). Interestingly, while the BAT ablated mouse is hyperphagic and obese (Lowell *et al* 1993), the UCP1 knockout mouse is not, and this mouse has a compensatory increase in UCP2 expression in BAT as well as in other tissues (Enerback *et al* 1997). Up-regulation of UCP2 mRNA occurs in mice resistant to diet-induced obesity (Surwit *et*

al 1998). Although UCP1 is expressed in *S. crassicaudata*, it does not appear to be important for thermogenesis (see chapter 13). If the function of UCP2 in *S. crassicaudata* is similar to that in rodents, then the finding that UCP2 mRNA expression fails to alter in response to changes photoperiod is consistent with observations that changes in physical activity, rather than changes in nutrient partitioning or thermogenesis, may be the primary mechanism by which alterations in adiposity occur.

Unlike most rodents, *S. crassicaudata* are heterothermic and utilise alterations in body temperature to conserve energy (see chapter 13). The failure of body temperature to alter in this study implies that *S. crassicaudata* are utilising mechanisms other than alterations in body temperature. Body temperature is not intended to act as a substitution for the measurement of metabolic rate.

The defence of fat mass by female *S. crassicaudata* during the shorter days of the year, when environmental temperatures are lower, is associated with a number of potential physiological advantages. In the wild, *S. crassicaudata* rely primarily on arthropods as a source of food. Arthropods are less abundant in colder months (Morton 1978c) and increased fat stores and/or lower energy requirements would, accordingly, optimise survival during winter. Similarly, maintenance of fat stores prior to breeding would allow females to expend less energy during the breeding season searching for food. Because food was available *ad libitum* in the current study, the magnitude of observed changes in tail diameter (and hence tail fat storage) may not be representative of what occurs in the wild, where animals are subjected to large

fluctuations abundance of prey. Although the laboratory diet given to the animals in this study is relatively high in fat (20% fat by dry weight), animals in the wild consume a high fat and high protein diet of arthropods. The observation that tail width increases with decreasing day-length in wild *S. crassicaudata* (Morton 1978c), however, suggests that the alterations in adiposity observed in this study are unlikely to be an artefact of the laboratory diet or conditions.

The increase in fat mass following ovariectomy in *S. crassicaudata* suggests that gonadal steroids are involved in the regulation of body fat stores in this marsupial, consistent with studies in other mammals such as rats, voles and guinea pigs (Dark *et al* 1984, Richard 1986, Slob *et al* 1973). Oestrogen was not replaced in this study, consequently it can not be determined whether the observed effects were due to alterations in oestrogen or another gonadal steroid. Evidence from other animal studies indicates a significant role for oestrogen in the regulation of body composition. For example, in rats oestrogen treatment prevents the increase in body weight observed following ovariectomy (Richard 1986) and in guinea pigs, oestrogen treatment reduces body weight (Slob *et al* 1973). Based on available data, it therefore seems unlikely that another gonadal steroid is responsible for the observed effects (Abelenda *et al* 1992). Increased fat mass in response to OVX occurred without significant alterations in energy intake; these findings are consistent with studies in Syrian hamsters demonstrating that the oestrous cycle is associated with substantial changes in body weight but not food intake (Schneider *et al* 1986), although in most rodents oestrogen has been shown to affect food intake (Wade *et al* 1985).

There a number of mechanisms by which oestrogen may affect body weight and adiposity. In rats oestradiol reduces lipoprotein lipase activity in WAT, and enhances thermogenesis in BAT (Wade *et al* 1985). In contrast, in the Syrian hamster alterations in ovarian steroid levels are not associated with changes in oxygen consumption or BAT thermogenesis (Schneider *et al* 1986). In rats, oestradiol has reduces the effect of noradrenaline in BAT (Fernandez *et al* 1994), suggesting that the response of other metabolically active tissues may be more important. In the current study, OVX had no effect on UCP2 mRNA expression in WAT in the LD animals, but a decrease in UCP2 mRNA was observed in the SD animals, which, interestingly, was the group with the greatest increase in fat mass. In LD OVX animals, UCP2 mRNA expression decreased only in skeletal muscle. The significance of these tissue specific effects are unknown, but there is evidence in rats that changes in UCP2 mRNA expression may occur in a tissue-specific manner in order to facilitate local energy supply (Dulloo *et al* 1998).

Very few studies have examined the effect of gonadal steroids on spontaneous physical activity. In cats, ovariectomy has no effect on physical activity despite substantial increases in food intake and body weight (Flynn *et al* 1996). In contrast, oestrogen administered centrally results in increased voluntary activity in rats (Wade *et al* 1985). The effect of gonadal steroids on physical activity in *S. crassicaudata* remain to be determined.

## CHAPTER 15

## CONCLUSIONS

The studies presented in this thesis have provided new data on the regulation of food intake, fat storage and energy balance in the dasyurid marsupial *S. crassicaudata*. The observation that *S. crassicaudata* stores approximately 25% of body fat in the tail, and that tail width is a sensitive indicator of total fat stores, characterises this animal as a novel and useful model to study the physiology of energy balance. Body fat stores of *S. crassicaudata*, as in other mammals, are physiologically regulated about a 'set-point', that is vigorously defended below a minimum, with a more flexible upper limit. At steady state, the 'set-point' is modified by alterations in gonadal steroids and photoperiod. Like most other mammals, *S. crassicaudata* expresses the uncoupling protein UCP1, but, in contrast to most other mammals, UCP1 is not used for the production of heat in brown adipose tissue. In this regard, despite significantly greater evolutionary separation, studies of thermogenesis in *S. crassicaudata* as opposed to rodents may be more comparable to adult humans, who similarly lack functional BAT. The homologous uncoupling protein UCP2, which is expressed in *S. crassicaudata* in a similar tissue distribution to rodents and humans, may be important in regulating energy balance in *S. crassicaudata*, most likely by regulating nutrient partitioning or thermogenesis. An increase in fat mass of *S. crassicaudata* following ovariectomy is associated with a decrease in UCP2 mRNA expression in skeletal muscle. A direct link between UCP2 and energy balance in *S. crassicaudata* remains to be established.

These studies have provided evidence that body fat stores of *S. crassicaudata* are primarily regulated by changes in energy expenditure and/or alterations in nutrient utilisation (*ie.* metabolic efficiency): i) after lipectomy, fat mass is restored without an increase in food intake, ii) a high calorie, high fat diet induces an increase in fat mass without increasing food intake, and iii) females housed under short-day photoperiods, and ovariectomised females, demonstrate an increase in fat mass without alterations in food intake.

Further evidence that *S. crassicaudata* can alter the thermogenic response and the partitioning of nutrients in order to maintain body composition in the face of a varying supply of calories and macronutrients comes from studies demonstrating i) that when fed isocaloric diets, an increase in dietary fat increases body fat stores of *S. crassicaudata*, despite a decrease in energy intake (Ng *et al* 1999), ii) an increase in dietary carbohydrate but not fat increases thermogenesis, and iii) the thermogenic response to food intake is dependent on the nutritional state (Clements *et al* 1998). This may represent a potential mechanism whereby fat mass of *S. crassicaudata* is increased when offered high fat, high calorie diets, without increased food intake. The role of macronutrient balance on body composition in *S. crassicaudata*, and the mechanisms by which this marsupial adjusts the oxidation of macronutrients to that consumed remains to be determined.

It is not known if the restoration of fat stores following lipectomy occurs by hyperplasia (proliferation of adipocytes at other fat depots) or hypertrophy (an increase in size of remaining fat cells). The defence of body fat stores, however, led to



the hypothesis that marsupials, like other mammals, would express the *ob* gene, and respond to leptin with a reduction in fat mass. The *S. crassicaudata* leptin gene was subsequently cloned, and shown to be highly homologous to that of other animals. Leptin mRNA expression in adipose tissue of *S. crassicaudata* correlates with fat mass, and leptin administration induced a decrease in food intake, body weight and fat mass. Although the function of leptin appears to be highly conserved, a high energy, higher fat, preferred diet induced resistance to the actions of leptin in *S. crassicaudata*. The failure of leptin to be effective in diet-induced obese animals suggests that leptin may be more important in defending a lower rather than an upper limit of body fat mass. Recent evidence obtained from this laboratory supports this conclusion; after diet-induced weight loss, leptin administration prevents restoration of fat mass in *S. crassicaudata*, despite consumption of a high calorie, higher fat diet, previously shown to induce weight gain and leptin resistance (Wittert *et al* 1999). It has not been established whether leptin affects physical activity of *S. crassicaudata*. Recent studies in this laboratory have demonstrated that leptin increases UCP2 mRNA expression in adipose tissue and liver (H Turnbull & G Wittert, personal communication), a finding consistent with a role for leptin to increase oxidation of free fatty acids.

The factors involved in the peripheral regulation of food intake are poorly defined. Food restriction decreases plasma glucose and increases plasma free fatty acid levels of *S. crassicaudata*. Energy intake, however, is not affected by changes in glucose utilisation. It appears that glucoprivic regulation of food intake may have evolved subsequent to the divergence of marsupials from the eutheria, representing an

alternative mechanism of appetite control. Although inhibitors of fatty acid oxidation increase food intake in some rodents, there is evidence to suggest that these inhibitors have tissue specific effects, which may explain discrepancies between studies. Alterations in free fatty acid oxidation are likely to play a minor role in regulating food intake in *S. crassicaudata*, although due to the inconclusive data obtained with mercaptoacetate this remains to be fully determined.

In order to maintain body composition, animals need to obtain food and eat a balanced diet under a range of adverse environmental conditions. Opioid peptides have been implicated as neurotransmitters involved in the choice of food in many animals, including invertebrates. As in other mammals, *S. crassicaudata* exhibits opioid modulation of both food intake and the choice of highly palatable foods. In *S. crassicaudata* these effects are mediated predominantly by the  $\mu$  opioid receptor. The observation that conservation of opioid mediated feeding occurs across such a wide variety of species supports the concept that endogenous opioids are of fundamental importance to the regulation of food intake.

During stressful conditions, animals need to minimise the energy expended in searching for food. The hypothalamic peptide CRF is important in the regulation of both the HPA axis and food intake in rodents; different CRF receptor subtypes are involved in mediating the HPA axis (CRF1 receptor) and food intake (CRF2 receptor) responses. In *S. crassicaudata*, peripherally administered CRF stimulates the release of cortisol, and centrally administered CRF decreases food intake. When administered peripherally, the CRF2 ligand, urocortin is more potent at reducing food intake in *S.*

*crassicaudata* than peripherally administered CRF. These findings suggest that, as with opioids, this mechanism of food intake is highly conserved and therefore fundamental in the regulation of food intake in all mammals.

In contrast to the highly conserved opioid feeding system, the gastrointestinally released peptides CCK, GLP-1 and bombesin appear to play no role in regulating food intake in *S. crassicaudata*, despite being tested at doses equivalent to or higher than those shown to be effective in rats. This is of interest since the marsupial CCK sequence is highly homologous to that of other mammals. These findings suggest that peptides released by the gastrointestinal tract may not represent highly conserved mechanisms of food intake. The actions of GRP to decrease food intake may be more highly conserved than some other peripherally released neurotransmitters.

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