Appetite and adiposity of the emu
(Dromaius novaehollandiae).

by

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Dedicated to

Jean Eiffler and Jim Graham

Who helped me choose a road less travelled.
Declaration

This thesis contains no experimental material that has been previously presented for any degree at any university or institution. The experimental designs and manuscript preparation were performed by myself, in consultation with my supervisors, Dr Dominique Blache, Dr Philip Vercoe and Professor Graeme Martin.

Gemma Graham

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Emus exhibit seasonal decreases in appetite and adiposity. Adipose tissue is the most profitable component of the carcass. As such, the reduction in adipose stores limits slaughtering to a brief window, and is a major constraint to productivity and industry development. To minimise the detrimental effects of seasonality on the productivity of farmed emus it is necessary to develop ways of manipulating appetite and adiposity. The general model for the control of appetite and adiposity is mediation of appetite and satiety centres in the hypothalamus by peripheral signals indicative of energy reserves and metabolic status. The relevance of this general model to the emu has not been investigated.

The general hypothesis in this thesis was that seasonal variation in appetite and adiposity in the emu operates under the general model developed in other species. The experimental models used to test the general hypothesis were increased appetite and adiposity (high fat diet, dexamethasone treatment), decreased appetite and adiposity (incubation) and increased appetite and decreased adiposity (starvation). To determine the role of leptin in the control of appetite and adiposity attempts were made to clone and sequence the emu leptin gene. The concentrations of metabolic hormones were measured in all of the experiments, and the role of NPY in the control of appetite and adiposity was investigated by quantifying the mRNA expression of NPY from the mediobasal hypothalamus and preoptic area in the glucocorticoid treatment, incubation and starvation experiments.

NPY mRNA expression did not change in response to dexamethasone treatment or starvation. There was no difference in NPY expression between incubating and non-incubating emus during the breeding season, despite large differences in their appetite and adiposity. Attempts to clone and sequence emu leptin were unsuccessful and further investigation of the relationship between leptin and NPY was not possible. However, it was demonstrated that emus have biologically active leptin by using a chicken leptin bioassay developed in Friedman-Einat's laboratory. High fat diets and dexamethasone treatment did not increase adiposity or food intake. Emus were relatively tolerant of starvation. Short-term starvation did not increase corticosterone concentration or NPY expression, or induce the classical starvation response in metabolic hormone concentrations that, in other species, would normally occur rapidly in response to
starvation. The hormone concentrations measured throughout these experiments were appropriate to the physiological requirements of the birds, and indicate that efficient mechanisms exist to defend the “set point” for energy reserves of the emu at a level appropriate to its needs and activities.

The fact that NPY gene expression is unchanged by starvation, incubation or dexamethasone treatment does not rule out a role for NPY in the hypothalamic control of appetite. Other appetite mediators such as, leptin or corticotrophin-releasing hormone (CRH), could, under the influence of photoperiod, inhibit NPY action or responses. Finally, it is apparent from the results that appetite and adiposity are tightly regulated in the emu. The emu also shows greater resistance to manipulation and tolerance of starvation than other species. These features make disrupting energy balance to increase adiposity a difficult enterprise for the farmer and, for the present, not feasible.
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General Introduction
The emu has evolved in a harsh environment and is able to contend with extremes of climate and food availability. A unique feature of the emu is its ability to fatten during the summer, a time when individual birds can dramatically increase their food intake and fat depot. As the oil produced from emu fat has proven therapeutic qualities there is the potential for emu farming to become a successful commercial venture. However, this potential is limited by the seasonal variation in appetite and subsequently adiposity, which prevents consistent market supplies of emu products. The industry could overcome these limitations if methods were developed to limit the decrease in food intake and reduction in the fat depot over the breeding season.

In other species, mainly mammalian, an integrated model for the control of energy balance has been developed. Metabolic hormones including, insulin, glucagon, thyroxine and triiodothyronine, and leptin, from adipose tissue, are peripheral factors that control energy balance through their effects on food intake and metabolic rate. One way that metabolic hormones keep energy intake and energy expenditure equal is by acting on neuropeptides involved in regulating appetite and satiety within the hypothalamus. One of the major neuropeptides involved in appetite control is neuropeptide Y (NPY), and one of the major regulators of NPY gene expression is leptin. The control of appetite and adiposity by NPY, leptin and peripheral hormones has not been investigated in the emu. As such, we do not know how well this model for the control of energy balance applies to the emu.

The aim of this thesis has been to improve the understanding of the control of appetite and adiposity in the emu. To address this aim, the general hypothesis of this thesis was that seasonal variation in appetite and adiposity in the emu operates under the general model developed in other species. The success of this study depends on how well the emu adheres to the general model for the control of appetite and adiposity. Given the strong evolutionary pressure for efficient use of available energy sources, the emu might have adopted particular strategies to control its energy balance. However, by comparing the emu to other species these strategies will be identified. In addition, it should be possible to develop methods for successfully manipulating appetite and adiposity to improve economic returns for the emu producer.
Chapter 1

Literature Review

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1. Introduction
The biggest problem facing the emu industry is decreasing adiposity due to reduced appetite during the breeding season. Adipose tissue is the major product of the emu. As such, improving our understanding of how appetite and fat deposition are controlled will enable the development of strategies to manipulate and maintain appetite and adiposity for longer periods of time. In this review I will cover the biology of the emu, and the history, constraints and potential of emu farming. Following on from this I will review the control of appetite and adiposity and their manipulation.

2. The emu
Emus belong to a group of flightless birds collectively called ratites. The mature emu stands up to two metres in height and weighs in the range of 30 to 50 kg. The feathers are grey-brown in colour, with a lighter ruff around the neck. The face and part of the throat is generally bare of feathers and grey-blue in colour, with females normally exhibiting darker colouring (Mawson, 1992). Following pairing, the breeding season commences in May and usually lasts until June, but can continue into October depending on seasonal conditions such as rainfall.

The onset of the breeding season occurs in response to decreasing day length (Blache & Martin, 1999; Blache et al, 2001a). The breeding season is associated with a 30-50% decrease in food intake and weight loss (Blake, 1996) (Fig. 1.1). The weight loss observed is due to appetite suppression resulting from several causes related to, the onset of the breeding season, broody behaviour by males while incubating eggs, and the egg laying period for the females. The female on average lays fifteen eggs in her first season and 22 eggs in subsequent seasons (O’Malley, 1991). The eggs are incubated by the male, or in incubators for 54 to 56 days (O’Malley, 1991). At the end of the breeding season appetite increases and weight returns to pre-breeding levels. In the incubating male this is particularly striking with a reversal from anorexia to a hyperphagic state. The hyperphagic state enables the males to regain most of the weight lost (24%) within a three-week period after the cessation of incubation (Van Cleeff, 2001).
Feed intake (kg/d) 10
Daylength (h) ~ 10

Figure 1.1: Annual changes in feed intake and live weight of male (open circles) and female (closed circles) emus (adapted from Blache et al, 2001b).
3. Emu farming

3.1 - History

Commercial emu farming commenced at Kalannie in Western Australia in 1970 (O'Malley, 1993). Although the first farm closed in 1973 it proved to other producers, and the government, that emus could be used successfully to produce high quality leather. On the basis of this success an experimental emu farm was set up at Wiluna by Applied Ecology, to be run by the Ngangganawili Aboriginal Community as both a tourism and leather production enterprise (O'Malley, 1993). Following this a private group, Dromaius Pty Ltd set up an experimental farm at Mount Gibson. The State Government recognised farming emus as 'technically feasible' in 1987 and approved the sale of 500 emu chicks from the Wiluna farm (O'Malley, 1993). By 1994 Western Australia boasted 42 operating emu farms producing emu meat, oil and leather (Smetana, 1994). The growth of the industry during the 1990's was indicative of the faith placed in it by producers and of the potential to utilise Australia's native fauna in ecologically sustainable production systems. The market has experienced a downturn in recent years with the commercial Australian flock numbering around 50,000 in 1999 (McKinna, 1999). Similar trends have been experienced in the other countries where producers enthusiastically entered into emu farming, among them New Zealand, America, China and parts of Europe. The downturn in emu numbers is the result of poor marketing of emu products (meat, leather and oil) in the wake of exponential increases in bird numbers, and high production costs.

3.2 - Production constraints

The relative youth of the industry means that much of the information required for a producer to decide on the best management practices is unknown for the emu. These unknowns include scientific knowledge of the bird’s nutritional requirements, the heritability of economically significant traits and the correlations between these traits, and how to manage birds to maximise production. At present the nutritional requirements and heritability of traits have been based on extrapolation of those for other avian species, usually the ostrich or chicken (O'Malley, 1996).

Another feature that has contributed to the emu industries problems is the high reproductive rate of emus, which resulted in an exponential increase in their numbers once farming commenced. The dramatic increase in bird numbers was problematic because the development of markets for emu products did not keep pace with the increase in bird numbers. Marketing groups still remain under a great deal of pressure to develop eager,
receptive markets. To achieve and maintain these markets it is recognised as essential for producers to reduce the costs of production by maximising productivity (Deeming & Angel, 1996).

Therefore, maintaining the viability of the emu industry is dependent upon both establishing stable markets for emu products and maximising productivity. Maximising productivity will reduce production costs and allow emu products to be more competitive in the market place. To maximise the returns per head it is essential to determine the optimal production conditions and methods for overcoming the utilisation of fat depots due to breeding behaviour. Furthermore, it will be important to prove to the market that consistent supplies of all products can be provided.

At present constant supplies of fat throughout the year are prevented by the weight loss that is associated with the commencement of the breeding season, this in turn limits the availability of meat and leather (Blake, 1996). In breeding animals the loss of fat is normal and acceptable. For animals destined for slaughter the loss of fat during the breeding season is undesirable, particularly when fat prices are high and fat is a significant component of the economic return obtained from a carcass.

It has been estimated that a 50 week old emu will yield 4kg of fat (Smetana, 1992). Long-term ex-farm prices have been estimated for the major emu products. Taking into account the costs of production, a long-term profit of $38 per bird has been estimated (Smetana, 1992). At present these profits are not being realised despite producers receiving, on average, higher prices for most products than the long-term predictions. The average prices received for 1999 in Australia were $26.38/L for oil, $62.50 per skin, $11.00/kg for meat, $4.75 per egg and $28.00 per live bird (Michael, 2000). The potential profits from emu farming have been eroded by the high cost associated with transport to abattoirs ($10-$40 per bird) and slaughter ($90-$100 per bird), leading to some producers opting to slaughter on farm and only sell the fat and leather (Michael, 2000). In addition to this producers have to transport birds on average more than 200kms to the nearest abattoir, as such they run a serious risk of damage to the birds’ skin during transport and a further reduction in profits (Michael, 2000).
3.3 - Industry potential

The long-term market trends are considered promising based on the public's initial response and the international interest that has been shown in emu products. The similarity of the leather produced to that of ostriches and rheas, and the ability to produce it in commercial quantities, makes targeting the markets for these other leathers a logical action. Other products such as carved eggs and jewellery crafted from feathers and nails are aimed at the tourist market, making their future returns difficult to estimate.

Emu meat makes up 30% of the carcass and is sold domestically and internationally (Dingle, 1997). It is a game meat derived from the thigh and pelvic area and has many healthy attributes (Berge et al, 1997). These include low levels of fat, cholesterol and calories (Dingle, 1997). In recent years emu meat has been awarded the Australian Heart Foundation tick of approval, a valuable and marketable endorsement. In addition, the fat that is present in emus is predominantly oleic acid, a mono unsaturated fatty acid, which is beneficial to human health (Dingle, 1997).

About 50% of the profits received from an emu come from fat, making it an important component of the carcass, especially as it makes up only 17.5% of the live weight (Dingle, 1997). The major consumers of fat are cosmetics companies, which use it as a base for cosmetics and therapeutic agents. Furthermore, the reputed therapeutic properties of emu oil have seen it accepted as an alternative, natural treatment for muscle and joint pain. The demand for emu oil for this purpose is likely to continue, particularly now that scientific support for its therapeutic properties has been obtained (Snowden & Whitehouse, 1997; Whitehouse et al, 1998; López et al, 1999). The scientific support for emu oils therapeutic properties has also allowed an application to be made for therapeutic goods approval (now pending) that will help to ensure the future of this product and the emu industry.

The problem of seasonal cycles of adiposity still remains a constraint to producers because it narrows the window of opportunity for slaughtering the birds. If this can be overcome emu farmers will have a better chance to establish markets for all their products and improve their profit margins. To do this it is essential that we increase our understanding of the control of appetite and fat deposition in the emu so that strategies to manipulate appetite and adiposity can be developed. To date no information exists regarding the control of appetite and adiposity in the emu. However, the literature contains a wealth of
information on the control of appetite and adiposity in other species and this can be used to identify a starting point for research in the emu.

4. Appetite and the regulation of adiposity

4.1 - The lipostatic theory

It has been proposed that an indirect calorimetry mechanism exists that regulates adipose reserves via sensitivity of the hypothalamus to the concentration of circulating metabolites (Kennedy, 1953). The lipostatic theory was developed on the basis of observations with rats that demonstrated they adjusted their food intake to very accurately maintain a constant fat mass and calorie intake (Adolph, 1947; Kennedy, 1950).

This theory was further supported by force-feeding experiments. Intragastrically cannulated dogs, fed diets corresponding to below, at and above their own determined requirements, adjusted their food intake such that the calorie intake was constant, and where possible at the original level (Janowitz & Hollander, 1955). Force-feeding rats and guinea pigs for prolonged periods above their requirements resulted in the development of obesity, whereas starved animals lost weight (Harris et al, 1986; Cohn & Joseph, 1962; Harris & Martin, 1984a & 1984b). In addition, when animals returned to ad libitum feeding, their food intake and body weight returned to the original level. This indicates that the regulation of food intake and appetite occurs via recognition of the fat store size and modification of food intake to maintain it at a “set point”.

4.2 - Control by the hypothalamus

The lipostatic theory, assumes that there are sites in the brain that receive signals pertaining to energy reserves and co-ordinate a response in terms of appetite, energy expenditure and metabolism. The hypothalamus is thought to be this site. Research has shown that damage to the hypothalamus of mammalian and avian species results in hyperphagia and consequently obesity (Brobeck, 1946; Lundbaek & Stevenson, 1947; Hervey, 1959; Anand, 1961; Lepovsky & Yasuda, 1965; Smith, 1968). In addition, decreased energy expenditure often contributes to the development of obesity in these animals as evidenced by a decline in the level of activity (Hetherington & Ranson, 1942; Brobeck, 1946).

The ventromedial region of the hypothalamus has been proposed as the site of action for appetite and adiposity control in the hypothalamus. This is based on the observations of Hetherington & Ranson (1940) that of discrete, localised lesions placed in almost all parts
of the hypothalamus, only those in the tuberal or posterior regions, and most effectively in the region of the ventromedial nuclei, induced obesity. Other research where obesity has been induced by creating bilateral lesions in the ventromedial region of the hypothalamus have supported Hetherington and Ranson's findings (1940), showing that such lesions cause the highest levels of obesity, primarily via increased food intake (Hetherington & Ranson, 1942; Brobeck, 1946; Kennedy, 1950; Hervey, 1959).

The findings of Brobeck (1946), Kennedy (1950) and Hervey (1959) indicated that initially, animals with lesions had an insatiable appetite that diminished with time but still remained above that of their intake prior to the operation. In the lesioning experiments of Hetherington & Ranson (1942) obesity occurred without increases in food intake. Instead, some animals returned to their previous level of food intake but displayed lower levels of activity than recorded prior to the procedure. This would indicate that the hypothalamus, as well as controlling appetite, might have some role in determining the energy expenditure of an animal based on its energy stores. There is also evidence that damage to the hypothalamus induces obesity via a shift in metabolism to favour fat deposition. For example, Van Putten et al (1955) pair fed rats with hypothalamic lesions to the level of unlesioned controls, which resulted in the pairs having identical body weights. However, carcass composition analysis revealed significantly higher proportions of fat in the lesioned animals.

4.3 - Fat deposition and mobilisation
The preceding sections have demonstrated that the size of the fat depot is regulated by the hypothalamus to maintain energy reserves at an appropriate level or "set point". When the energy reserve is too low, appetite is increased and adipose sparing mechanisms are initiated, and when the energy reserve exceeds requirements the animal feels sated and mechanisms to increase energy expenditure may be initiated. Research since the proposition of the lipostatic theory has demonstrated that these mechanisms rely on the interaction of hormones, metabolites and signals from the digestive tract (Fig. 1.2).

In the gastrointestinal tract enzymes convert carbohydrates to monosaccharides, fats to fatty acids and glycerol and proteins into amino acids (Duke, 1986). These enzymes are released in response to food consumption and digestion. Cholecystokinin, a hormone originating from the upper small intestine, is released in response to duodenal distension, vagal stimulation and the presence of nutrients from food (Duke, 1986). Elevation of
cholecystokinin concentrations decreases food intake via central actions (Savory & Gentle, 1980; Denbow & Myers, 1982; Denbow, 1986).

Nutrients absorbed from the gastrointestinal tract enter the hepatic portal blood system and are transported to the liver. Elevations of glucose, amino acid or fatty acid concentrations decrease food intake (Denbow, 1999). Glucose decreases food intake via chemoreceptors in the liver that act on the central nervous system (Forbes, 1988). The lipid component of the absorbed nutrients is converted to very low-density lipoproteins (VLDL's) in the liver, and transported to peripheral tissues (Stevens, 1996). Lipoprotein lipase hydrolysates the VLDL, releasing fatty acids that are taken up by tissues to meet their energy requirements or stored in adipose tissue (Stevens, 1996).

![Figure 1.2: A model for the control of food intake and adipose tissue deposition. The blue lines indicate stimulation (solid) or inhibition (dashed) of hormone release or production. The black and red lines indicate negative (dashed) or positive (solid) effects. CCK = cholecystokinin, CRH = corticotrophin-releasing hormone, FFA = free fatty acid, NPY = neuropeptide Y.](image_url)

The storage or mobilisation of energy reserves is controlled by the interactions of hormones. The most important hormones in the storage and mobilisation of energy reserves are insulin, glucagon, glucocorticoids, catecholamines and leptin. In mammals,
insulin is the primary regulator of metabolism. Insulin stimulates the uptake and storage of lipids. In avian species, it does this by acting on the liver to increase glucose uptake, inhibit gluconeogenesis, and increase lipogenesis and the release of VLDL from hepatocytes (Stevens, 1996). In avian adipose tissue, insulin increases glucose uptake, inhibits free fatty acid release, stimulates the conversion of glucose to fat and stimulates lipoprotein lipase concentrations (Griminger, 1986; Hazelwood, 1986). These actions of insulin spare the triglyceride stores and increase adipose deposition. In mammals, insulin acts centrally to decrease appetite via suppression of neuropeptide Y (NPY) gene expression (Schwartz et al, 1991 & 1992). It is likely that this also occurs in avian species, and provides a negative feedback loop to maintain energy reserves at the correct level.

In contrast to mammals, glucagon is the primary regulator of metabolism in avian species. Glucagon mobilises energy reserves by inhibiting lipogenesis and stimulating gluconeogenesis in the liver and stimulating lipolysis in adipose tissue (Stevens, 1996). The increased lipolytic activity of adipose tissue releases free fatty acids into the peripheral circulation (Braganza et al, 1973). Glucagon release from the pancreas is stimulated by insulin, free fatty acids and cholecystokinin and profoundly inhibited by glucose, providing positive and negative feedback loops (Hazelwood, 1986).

Catecholamines are released from the adrenal gland in response to signals from the sympathetic nervous system (Stevens, 1996). The catecholamines mobilise stored energy reserves primarily from the liver, and to a lesser extent from adipose tissue, by stimulating lipolysis and inhibiting fatty acid synthesis (Stevens, 1996). Glucocorticoids are released from the adrenal gland in response to increasing adrenocorticotropic (ACTH) concentrations in the anterior pituitary (Stevens, 1996). The increase in the concentration of ACTH is in turn controlled by corticotrophin-releasing hormone (CRH) at the hypothalamic level (Laycock & Wise, 1996; Kacsoh, 2000). The glucocorticoids have opposing actions in liver and adipose tissue, stimulating lipogenesis and gluconeogenesis in the liver, and lipolysis in adipose tissue (Griminger, 1986; Stevens, 1996). Glucocorticoids also have a negative feedback function, suppressing hypothalamic CRH production (Kacsoh, 2000). Elevations in CRH, as well as increasing glucocorticoid release, also suppress the actions of NPY to increase appetite (McCarthy et al, 1993; Menzaghi et al, 1993). This action of CRH on NPY is proposed as one of ways that depressed appetite is maintained in humans suffering from anorexia nervosa (Støving et al, 1999).
CRH is regulated by leptin, a hormone secreted by adipocytes (section 5.1). Leptin increases the hypothalamic CRH content and expression in vivo, and CRH secretion in vitro (Schwartz et al, 1996a; Costa et al, 1997; Uehara et al, 1998). In rats, pre-treatment with antibodies against CRH attenuates the ability of leptin to decrease appetite (Okamoto et al, 2001). As such, CRH may be involved in the suppression of NPY expression and appetite following central leptin administration (Jang et al, 2000). The role of leptin in avian metabolism has not been extensively studied and is complicated by doubts over the veracity of published sequences for the chicken leptin gene (see section 5.1 for further discussion). The evidence to date indicates that leptin expression in the liver, but not adipose tissue, increases in response to insulin administration (Ashwell et al, 1999a; Richards et al, 1999). In mammals, the expression of the leptin gene, and leptin secretion, are stimulated by insulin (Saladin et al, 1995; Barr et al, 1997). Insulin and leptin both act centrally to decrease appetite (Mistry et al, 1997; Tang-Christensen et al, 1999).

There is evidence that leptin increases the sensitivity of animals to the satiety effects of cholecystokinin (Emond et al, 1999; Matson & Ritter, 1999). Furthermore, leptin increases the metabolic rate and energy expenditure (Pelleymounter et al, 1995; Collins et al, 1996; Hwa et al, 1996; Hwa et al, 1997; Mistry et al, 1997). Both metabolic rate and energy expenditure are disrupted, along with appetite, following hypothalamic lesions (Hetherington & Ranson, 1942; Brobeck, 1946; Van Putten et al, 1955). As such, leptin is a powerful regulator of adipose reserves at the hypothalamic level, via its ability to alter appetite, energy expenditure and metabolism, in response to changes in energy balance.

The action of leptin to alter appetite, energy expenditure and metabolism at the hypothalamic level is mediated by the interaction of a large number of neuropeptides. NPY is one of these neuropeptides, it has been mentioned above and has been investigated in the course of this thesis. NPY is discussed further in section 5.2. Other hypothalamic neuropeptides that have a role in the control of appetite, energy expenditure and metabolism include agouti-related protein, cocaine and amphetamine-regulated transcript, orexin/hypocretin, proopiomelanocortin and CRH (McCarthy et al, 1993; Menzaghi et al, 1993; Mizuno et al, 1997; Ollmann et al, 1997; Sakurai et al, 1998; Heinrichs & Richard, 1999; Vrang et al, 1999). However, these neuropeptides were not investigated in the course of this thesis. Instead, my investigations have focused on NPY as it has been
extensively studied in avian species and is considered to be a major central pathway involved in the control of appetite, energy expenditure and metabolism.

5. Understanding the control of appetite and adiposity in the emu
Hormones, metabolites and genes interact to control appetite via their response to food intake, absorption of nutrients and changes in the size of the adipose depot (Fig. 1.2). In the following sections those hormones, metabolites and genes that were investigated in the course of this thesis are discussed further.

5.1 - Leptin
In the last decade the leptin gene has been cloned and sequenced for a number of species (Zhang et al, 1994; Masuzaki et al, 1995; Murakami & Shima, 1995; Ramsay et al, 1998). The gene encodes a 4.5 kilobase adipose tissue specific messenger RNA (mRNA) in both mice and humans (Zhang et al, 1994; Masuzaki et al, 1995). There is 84% homology between the human and mouse leptin genes. The product of this gene, leptin, is a satiety signal involved in the regulation of appetite and adiposity (Friedman, 1996; Sinha, 1997; Auwerx & Staels, 1998; Buchanan et al, 1998; Inui, 1999b). The concentration of leptin corresponds closely to body mass index (BMI), a correlate of adiposity (Maffei et al, 1995; Considine et al, 1996; McGregor et al, 1996; Rosenbaum et al, 1996; Schwartz et al, 1996b; Niskanen et al, 1997; Tataranni et al, 1997; Parry et al, 1998; Jensen et al, 1999).

In non-human species leptin concentrations and leptin mRNA expression are directly correlated with adiposity (Frederich et al, 1995; Maffei et al, 1995; De Schepper et al, 1998; Hissa et al, 1998; Robert et al, 1998).

Zhang et al (1994) hybridised the leptin gene to Southern blots of genomic DNA from the mouse, rat, rabbit, vole, cat, cow, sheep, pig, human, chicken, eel and fruit fly. For all the vertebrate species, detectable signals were present, indicating that they contained DNA homologous to the mouse leptin gene. The presence of a signal in the chicken indicates that the leptin gene is likely to be present in other avian species, including emus.

The controversy surrounding the cloning of the avian leptin gene.
Support for the presence of the leptin gene in avian species has been obtained from the sequencing of the chicken leptin receptor (Horev et al, 2000). Two groups also claim to have cloned the chicken leptin gene (Taouis et al, 1998; Ashwell et al, 1999a & 1999b). Subsequently, a recombinant chicken leptin has been produced (Raver et al, 1998). It is
claimed that the chicken sequence shares a high degree of homology (95-97%) with the mouse leptin gene (Taouis et al, 1998; Ashwell et al, 1999a). However, the chicken leptin receptor shares only a low degree of homology with mammalian sequences (on average 60%), and this casts doubt on the claims to have sequenced the chicken leptin gene as receptor sequences are normally more highly conserved than the gene (Horev et al, 2000). Further doubt is cast by the maintenance of appetite in chickens receiving central injections of the highly homologous (95-97%) murine recombinant leptin (Bungo et al, 1999). This is an unexpected finding given that human recombinant leptin, despite being less homologous to the murine sequence (84%), can decrease appetite when administered to the mouse (Hwa et al, 1996). Southern hybridisation has demonstrated that the homology between mouse and chicken leptin genes cannot be more than 72-77% (Varma, 2000). This degree of homology is much lower than the 95-97% homology at the nucleotide level claimed by Ashwell et al (1999a) and Taouis et al (1998). Northern hybridisation with a mouse leptin probe to chicken adipose RNA failed to produce a signal, and PCR and RT-PCR using primers designed for the mouse and the published chicken leptin sequences also failed to generate PCR products (Friedman-Einat et al, 1999). Subsequently, claims to have successfully cloned the chicken leptin gene have been openly challenged by avian researchers (Dunn et al, 2001; Varma, 2000; Horev et al, 2000; Friedman-Einat et al, 1999).

Mechanism of action of leptin

Daily administration of recombinant leptin to mice lacking functional leptin (ob/ob genotype) causes time and dose dependent reductions in body weight and food intake (Weigle et al, 1995; Tartaglia et al, 1995; Pelleymounter et al, 1995; Halaas et al, 1995; Campfield et al, 1995; Stephens et al, 1995; Hwa et al, 1996). In normal mice, recombinant leptin reduces food intake, even after 24 hours of fasting (Pelleyounter et al, 1995; Rentsch et al, 1995; Mistry et al, 1997). The action of leptin to decrease body weight does not occur purely via appetite suppression, as the food intake of normal mice treated with leptin returned to its original level without their body weight returning to pre-injection levels. Other changes attributed to leptin administration include alterations in metabolism, level of activity, oxygen consumption, and the normalisation of body temperature, serum insulin and glucose levels in the ob/ob mouse (Pelleyounter et al, 1995; Hwa et al, 1996; Hwa et al, 1997; Mistry et al, 1997).

Collins et al (1996) proposed that leptin regulates the extent of adipose tissue reserves by a
mechanism that involves both appetite suppression and sympathetic nervous system signaling to increase thermogenesis and energy expenditure in brown adipose tissue as fat mass increases. To test this hypothesis leptin was administered to ob/ob mice and their noradrenaline turnover was measured after blocking catecholamine synthesis, which is indicative of the sympathetic outflow. The administration of leptin caused a selective increase in noradrenaline turnover in brown adipose tissue, with no effect upon turnover in white adipose tissue. Taken together these findings demonstrate that leptin regulates body weight via effects on both food intake and the level of energy expenditure.

5.2 - Neuropeptide Y
Leptin's role in the regulation of energy reserves is as a signal of the adipose depot size, that initiates appropriate responses in terms of appetite, energy expenditure and metabolic rate in response to deviations from the "set point". One way in which leptin achieves this is via its effects on neuropeptide Y (NPY), a potent appetite stimulant. There is an inverse relationship between the expression of leptin and NPY.


The food intake of animals is higher once food is returned after a period of fasting. This mirrors the increase in feed intake following central NPY administration, and research indicates that endogenous NPY mediates the feeding responses provoked by food deprivation (Stanley et al, 1992; Bivens et al, 1998). In addition, it has been found that passive immunisation against NPY markedly suppresses feed intake in non-fasted rats (Dube et al, 1994). It is apparent from these results that NPY is an important signal
stimulating ingestive behaviour.

In addition to its effects on appetite, NPY promotes adipose deposition by decreasing energy expenditure and thermogenesis, and increasing the efficiency of energy storage (Roscoe & Myers, 1991; Billington et al, 1994; Currie & Coscina, 1995; Stephens et al, 1995; Woods et al, 1998). These actions of NPY decrease the metabolic rate but do not appear to involve modulation of the thyroid, as NPY injection has no effect on thyroid gland weight, or the serum concentrations of thyroid stimulating hormone, triiodothyronine and thyroxine (Malendowicz & MiSkowiak, 1990). NPY does however increase the consumption of carbohydrate preferentially to fats or protein (Stanley et al, 1985b; Morley et al, 1987). NPY also increases the respiratory quotient, indicating that carbohydrate catabolism is increased (Menéndez et al, 1990). As a consequence of this, fat synthesis is increased.

NPY also interacts with metabolic hormones to promote adipose deposition and energy sparing mechanisms. Elevations in NPY expression increase the level of circulating insulin (Walker & Romsos, 1993; Billington et al, 1994; Tomaszuk et al, 1996; Parikh & Marks, 1997). This favours fat deposition via increased triglyceride synthesis and deposition in white adipose tissue (Stevens, 1996). As such, prolonged elevation of NPY induces obesity, via increased appetite and metabolic changes that favour fat synthesis and deposition (Stanley et al, 1986). The effect of NPY to increase the concentration of insulin may also function as a negative feedback loop reducing NPY expression and preventing excessive adipose deposition. This idea is supported by research demonstrating that the central administration of insulin decreases NPY mRNA expression in rats (Schwartz et al, 1991 & 1992). In rats, without access to food, the central administration of NPY increases the concentration of glucagon and causes a transient increase in plasma glucose without effecting the concentration of corticosterone (Marks & Waite, 1996). Central NPY administration also decreases insulin sensitivity (Marks & Waite, 1997). During fasting, the effects of NPY to increase the concentration of glucagon and decrease insulin sensitivity interfere with the control of glucose metabolism by insulin. Hypothalamic NPY content is rapidly increased in avian and mammalian species by food deprivation or restriction (Chua et al, 1991; Sahu et al, 1992; Marks et al, 1992; McShane et al, 1993; Davies & Marks, 1994; Stricker-Krongrad et al, 1997; Jang & Romsos, 1998; Boswell et al, 1999; Mizuno et al, 1999; Boswell, 2001; Kameda et al, 2001). This elevation in NPY is associated with decreased insulin and glucose and increased corticosterone
concentrations (Marks et al., 1992; Mercer et al., 1996; Jang & Romsos, 1998; Dallman et al., 1999; Mizuno et al., 1999).

Role of NPY in the seasonal cycle of food intake
In some species seasonal cycles of food intake and fat metabolism are observed. These are proposed to occur via a “sliding set point” for homeostasis (Mrosovsky, 1976; Mrosovsky & Sherry, 1980; Mercer, 1998). The mechanisms that control this are not well understood, and the role of NPY is not clear. In sheep, lower levels of NPY expression are associated with periods of low appetite (Clarke et al., 2000). Siberian hamsters, a hibernating species, also have lower levels of NPY mRNA expression during short photoperiods, when appetite is low (Reddy et al., 1999). In addition, this experiment demonstrated that 48 hours of food deprivation, during both short and long photoperiods, increased NPY mRNA expression (Reddy et al., 1999). Furthermore, the Siberian hamster increases its food intake in response to the central administration of NPY, on both long and short photoperiods (Boss-Williams & Bartness, 1996). Another hibernating species, the golden mantled ground squirrel, also increases its food intake in response to central NPY infusion (Boswell et al., 1993). It can be inferred from these findings that NPY expression is correlated with appetite, and that the responsiveness of central pathways to NPY’s actions are not impaired by changes in photoperiod.

It is likely that the “sliding set point” for homeostasis applies to avian species as well, via similar mechanisms. In the white-crowned sparrow, as with the Siberian hamster, central administration of NPY increases food intake on both short and long days (Richardson et al., 1995). This experiment also demonstrated that during long days, when food intake is increased, the animals were more sensitive to the effects of NPY on appetite (Richardson et al., 1995). As such, a plausible mechanism for photoperiod induced changes in the “sliding set point” for homeostasis is altered sensitivity to central appetite mediators.

5.3 - VIP, prolactin and reproduction in birds
Changing photoperiod increases the expression of vasoactive intestinal polypeptide (VIP) and prolactin secretion that are involved in the induction and maintenance of incubation behaviour in birds. VIP was isolated from porcine small intestine (Said & Mutt, 1970). It has since been isolated and characterised for a range of species including chickens, cattle, humans and rats (Nilsson, 1974 & 1975; Carlquist et al, 1979 & 1982; Dimaline et al., 1984). VIP acts as a vasodilator in the periphery, stimulates respiratory chemoreceptors,
increases cardiac output and induces hypotension and hyperglycemia (Said & Mutt, 1970).

VIP is widely distributed throughout the central nervous system, and is found at highest levels in the hypothalamus (Bryant et al, 1976; Said & Rosenberg, 1976; Yamada et al, 1982; Kuenzal et al, 1997; Chaiseha & El-Halawani, 1999). Elevations of VIP stimulate prolactin release in birds and mammals (Kato et al, 1978; Fuxe et al, 1979; Macnamee et al, 1986; Opel & Proudman, 1988; Sharp et al, 1989; Pitts et al, 1994; Maney et al, 1999; Vleck & Patrick, 1999). In avian species the rise in VIP, and subsequently prolactin, is involved in the induction and maintenance of broody behaviour (El Halawani et al, 1995; Crisóstomo et al, 1997; Bédécarrats et al, 1999; Sockman et al, 2000). In birds immunised against VIP, photo-stimulation fails to induce a rise in prolactin or induce incubation behaviour, demonstrating that VIP is an essential intermediary in prolactin response to photoperiod and for the induction of incubation (El Halawani et al, 1996; Crisóstomo et al, 1997).

5.4 - Metabolic and hormonal consequences of incubation

The incubation period in birds can be divided into three phases (Le Maho et al, 1981; Castellini & Rea, 1992). In the goose, phase I occurs in the first few days of incubation (Le Maho et al, 1981). Phase I involves a shift from metabolism of ingested nutrients to meet energy requirements to utilisation of the adipose stores, with minimal weight loss. Phase II from day 3 to 30 of incubation is associated with a reduced resting metabolic rate and complete reliance on adipose stores for energy requirements. The third phase from day 30 until the completion of incubation involves protein breakdown to supply the animals energy requirements as the lipid stores are depleted. The hormonal changes associated with incubation decrease the metabolic rate and spare the animals energy reserves. These changes include decreased thyroxine and insulin, decreased or unchanged triiodothyronine concentrations and increased corticosterone and glucagon concentrations (Chieri et al, 1972; Cherel et al, 1988; Groscolas & Leloup, 1989; Van Cleeff, 2001). The incubation period is also associated with depressed appetite in many avian species (Mrosovsky, 1976; Savory, 1979; Mrosovsky & Sherry, 1980; Zadworny et al, 1985; Cherel et al, 1988).

Prolactin is elevated during incubation and has been shown to have variable effects on appetite. In mammalian species, food intake can be increased, decreased or remain unchanged when prolactin is suppressed (Shani et al, 1975; Brien, 1986; Bartness et al, 1987; Curlewis et al, 1988 & 1991; Salah et al, 1995). In both mammalian and avian
species, appetite increases, decreases or does not change in response to prolactin administration (Shani et al, 1975; Ryg & Jacobsen, 1982; Denbow, 1986; Moore et al, 1986; Bartness et al, 1987; Curlewis et al, 1988; Buntin & Figge, 1988; Gerardo-Gettens et al, 1989a & 1989b; Heil, 1999; Ebenezer & Parrott, 1991; Byatt et al, 1993; Hnasko & Buntin, 1993; Noel & Woodside, 1993; Salah et al, 1995; Buntin et al, 1999). In ring doves the increase in appetite in response to prolactin administration occurs via an increase in the number of cell bodies that are immunoreactive to NPY (Strader & Buntin, 2001). However, despite elevated prolactin concentrations, appetite is not increased during incubation. It is possible that differential regulation of appetite occurs in the central nervous system and is dependent on reproductive status, factors such as the egg or nest stimuli, and the permissive action of other hormones (Buntin & Tesch, 1985; Janik & Buntin, 1985; Horseman & Buntin, 1995). This differential regulation could take the form of an inhibition of NPY’s response to elevated prolactin levels, or inhibition of NPY’s actions to increase appetite during the reproductive period.

6. Manipulation of appetite and adiposity

The decreased appetite and adiposity observed during incubation is an adaptation to enable a more important process, namely reproduction, to succeed. It occurs via endogenous disruption of the mechanisms controlling energy balance. The control of appetite and adiposity can also be manipulated exogenously, to prevent the animal from maintaining energy reserves at the correct “set point”. Manipulating the “set point” can be achieved either by physical means (i.e. starvation or force-feeding) or by interfering with the animal’s mechanisms of assessment and response to changes in the extent of the energy reserves (i.e. hypothalamic lesions, high fat feeding, glucocorticoid treatment). The manipulations employed during the course of this experiment are discussed below.

6.1 - High fat diets

The degree of adiposity induced by a high fat diet is proportional to the diet's fat content (Edozien & Switzer, 1978; Deaton et al, 1981; Boozer et al, 1995; Vilà & Esteve-Garcia, 1996). High fat diets can increase adiposity without increasing energy consumption or body weight (Oscai et al, 1984; Storlien et al, 1986; West et al, 1992; Boozer et al, 1995; Smith et al, 1998). They are able to do this because more calories are obtained from fat, and provided the oxidation of carbohydrates and proteins is sufficient to meet energetic requirements, fats are stored.

High fat diets increase fat consumption and storage via the following mechanisms:

1. Lower satiety of diets high in fat (French et al, 1995; Covasa & Ritter 1998 & 1999; Romon et al, 1999; Havel et al, 1999),
2. Greater absorbance of fat from the diet than protein or carbohydrate (Singh et al, 1972; Reed et al, 1991; French et al, 1995),
3. Slower induction of fat oxidation from the diet than protein or carbohydrate (Flatt et al, 1985; Hill et al, 1992; Prentice, 1998; Friedman, 1998), and

Because of these mechanisms, particularly the lower satiating effect, consumption of high fat diets can lead to passive over consumption of energy and increased adiposity (Bray & Popkin, 1998).

In mice, high fat diets induce peripheral, but not central, leptin resistance, and the onset of resistance is faster the higher the diets fat content (Van Heek et al, 1997). It has been proposed that leptin is a modulator of weight gain that is overridden by the continuous presence of high levels of fat in the diet (Ahrén, 1999). Such a mechanism allows animals to take full advantage of high fat food sources when they are available and thereby increase their chances of surviving subsequent famines. The ability of mice to override the control of appetite and adiposity by leptin suggests that they have a “thrifty” genotype. The “thrifty” genotype was proposed by Neel (1955) as an explanation of the greater efficiency of food utilisation associated with inherited metabolic disorders. These genotypes have evolved because greater efficiency of food utilisation is an advantage in famine situations and allows for greater storage of ingested nutrients. However progress has eliminated feast and famine situations in the Western world and along with the high fat content of today’s diet has rendered this “thrifty” genotype detrimental to health. It seems logical that other
species have developed similar strategies for coping with feast and famine situations. The emu is subjected to extremes of food availability in the wild and it is probable that it has evolved mechanisms to take full advantage of abundant food sources and increase adipose deposition.

It is likely that these mechanisms may involve metabolic adaptations, however these can vary widely between species and strains. Rats fed high fat/protein diets had increased fasting plasma glucose concentrations, glucose intolerance and insulin resistance (Ramirez et al., 1990). In other experiments, rats fed diets that contained from 12 to 48% of energy as fat, did not show differences in their insulin or glucose concentrations (Boozer et al., 1995). In mice, glucose and insulin concentrations are unchanged by high fat diets although insulin sensitivity is reduced (Lemonnier et al., 1975). However, other researchers have found that high fat diets (58%) increase insulin concentrations, and create a state of insulin resistance and glucose intolerance (Ahrén & Scheurink, 1998).

6.2 - Glucocorticoids

As with high fat diets, the actions of glucocorticoids to increase adiposity result from hormonal changes that alter metabolism (Fig. 1.3). Glucocorticoid treatment increases circulating insulin concentrations in avian and mammalian species (Campbell et al., 1966; Simon, 1984; McKibbin et al., 1992; Corah et al., 1995; Dallman et al., 1995; Berneis et al., 1996; Miell et al., 1996; Makino et al., 1998; Zakrzewska et al., 1999). In birds, increased concentrations of insulin favour adipose deposition (Griminger, 1986). A feature of chronic glucocorticoid treatment is increased adiposity in both avian and mammalian species (Hausberger & Ramsay, 1953; Dulin, 1956; Nagra & Meyer, 1963; Nagra, 1965; Simon, 1984; Kafri et al., 1988; Corah et al., 1995). This arises from the induction of insulin resistance and the action of concomitant elevations of insulin and glucocorticoids to increase adipose deposition (McKibbin et al., 1992; Dallman et al., 1993; Taouis et al., 1993; Dallman et al., 1995). The increased adiposity observed with glucocorticoid treatment is not necessarily associated with increased appetite or live weight.

In both birds and mammals, glucocorticoid treatment may be accompanied by decreases in both appetite and weight gain (Greenman & Zarrow, 1961; Simon, 1984; Kafri et al., 1988; McKibbin et al., 1992; De Vos et al., 1995; Makino et al., 1998; Zakrzewska et al., 1999). As central insulin administration decreases neuropeptide Y (NPy) mRNA expression, the increase in insulin concentration associated with chronic dexamethasone treatment may
contribute to appetite depression (Schwartz et al, 1991; Schwartz et al, 1992). Indeed, in rats, NPY was increased in the lateral hypothalamic area following acute but not chronic dexamethasone treatment (McKibbin et al, 1992). In this experiment insulin was not altered 4 hours after an injection of dexamethasone, but was increased after 7 days of treatment and associated with decreased appetite (McKibbin et al, 1992). In dogs, chronic dexamethasone treatment impairs insulin transport into the central nervous system (Baura et al, 1996). As such, it would seem unlikely that insulin could suppress hypothalamic NPY expression in these animals. In Syrian hamsters, chronic dexamethasone treatment increased NPY expression and depressed weight gain without changing the insulin concentration (Mercer et al, 1996). These conflicting results represent species-specific differences in response to dexamethasone, and highlight the need for further research in this area.

The trend for decreased weight gain indicates that glucocorticoids have catabolic effects. These catabolic effects are particularly pronounced at high doses and result from the bitonic response of live weight and feed efficiency to glucocorticoids (Devenport et al, 1989; Dallman et al, 1993). This response is due to the presence of two corticosterone receptors, type I and type II. With increasing glucocorticoid concentrations the type II receptor is stimulated to a greater extent, promoting tissue catabolism (Devenport et al, 1989). The synthetic glucocorticoid dexamethasone, in contrast to other glucocorticoids, only stimulates the type II receptor. Despite this dexamethasone treatment can increase appetite and adiposity (Adams & Sanders, 1992; Corah et al, 1995). This is because the increase in insulin observed with dexamethasone treatment promotes adipose deposition, and contributes to increases in the fat to lean mass (Devenport et al, 1989; Corah et al, 1995; Miell et al, 1996; Kolaczynski et al, 1997).

Glucocorticoids have variable effects on glucose concentrations in both avian and mammalian species (Greenman & Zarrow, 1961; Campbell et al, 1966; Simon, 1984; Joseph & Ramachandran, 1992; McKibbin et al, 1992; Papaspyrou-Rao et al, 1997; Makino et al, 1998). In birds, increased concentrations of glucose have a sparing effect on adipose stores, via decreases in the concentration of glucagon and adipose utilisation (Hazelwood, 1986). Another feature of glucocorticoid treatment, in both birds and mammals, is increased liver weight and liver fat contents (Dulin, 1956; Campbell et al, 1966; Adams, 1968; Simon, 1984, Kafri et al, 1988). The increased liver weight is due to an accumulation of fat in the liver because of the action of glucocorticoids to promote
gluconeogenesis and lipogenesis in this tissue (Dallman et al, 1993). These actions increase the extent of the energy reserves.

Figure 1.3: The hypothalamic-pituitary-adrenal axis: effects of endogenous (upper diagram) and exogenous (lower diagram) glucocorticoids (solid lines indicate stimulation/increase, dashed lines indicate inhibition/decrease). Grey lines and labels indicate that effects are diminished or unknown. CRH = corticotrophin-releasing hormone, POMC = proopiomelanocortin (precursor for ACTH), ACTH = adrenocorticotropic hormone. Adapted from Kacsoh (2000).
In the chicken, acute dexamethasone has variable effects on plasma triiodothyronine and decreases plasma thyroxine concentrations (Mitchell et al, 1986; Darras et al, 1997). These effects on thyroid hormone concentration are due to inhibition of thyroid stimulating hormone release, decreases in the release of thyroid releasing hormone (TRH) and a diminished expression of proTRH mRNA (van Haasteren et al, 1996; Darras et al, 1997). These changes in thyroid hormones could be expected to decrease the metabolic rate and have a sparing effect on adipose stores. Indeed acute dexamethasone treatment decreases heat production in chickens, indicating a decreased metabolic rate (Mitchell et al, 1986).

Glucocorticoids also impact on reproductive function in birds. In chickens, corticosterone administration induced ovarian regression and decreased the concentration of luteinising hormone, progesterone and oestradiol (Etches et al, 1984). Acute dexamethasone treatment decreased luteinising hormone secretion and prevented ovulation when given 13-14 hours before the expected time of ovulation (Soliman & Huston, 1974; Wilson & Lacassagne, 1978). This effect is due to the action of dexamethasone to inhibit ACTH release and suppress corticosterone secretion (Arimura et al, 1969; Sirett & Gibbs, 1969; Buckland et al, 1974; Etches, 1976; Arvat et al, 1998). The suppression of corticosterone secretion prevents the positive feedback of corticosterone on luteinising hormone secretion (Wilson & Lacassagne, 1978).

Clearly, manipulations of the hypothalamic-pituitary-adrenal axis (HPA) by glucocorticoid administration can alter the metabolic rate and hormone concentrations to favour increases in adiposity. As such, treating emus with glucocorticoids provides an opportunity to improve our understanding of the control of appetite and metabolism, while determining the potential of glucocorticoids as a means of increasing adiposity in the emu.

6.3 - Starvation
In contrast to treatment with high fat diets or glucocorticoids, the effects of starvation on appetite and adiposity are clear. Starvation increases appetite, decreases adiposity and initiates mechanisms to conserve energy reserves. These mechanisms include decreased energy expenditure and reductions of the metabolic rate, and are paralleled by changes in metabolic hormones. In mammals, starvation induces rapid changes in the concentration of metabolic hormones, including:

1. Decreased insulin (Lyle et al, 1984; Nair et al, 1987; Webber & Macdonald, 1994; Mercer et al, 1998; Dallman et al, 1999),
2. Increased glucagon (Stout et al, 1976; Goschke & Tholen, 1977; Nair et al, 1987),
3. Increased corticosterone (Dallman et al, 1999),
4. Decreased glucose (Stout et al, 1976; Lyle et al, 1984; Nair et al, 1987; Webber & Macdonald, 1994; Dallman et al, 1999),
7. Decreased thyroid stimulating hormone (Harris et al, 1978; Ikeda et al, 1991)
and,
8. Increased growth hormone (Goschke & Tholen, 1977).

Avian species respond to starvation in a similar manner to mammalian species. However, they differ from mammalian species in that glucagon rather than insulin is the primary regulator of metabolism, especially during food deprivation (Hazelwood, 1986). In birds voluntary or imposed starvation induces the following changes in the concentration of metabolic hormones:

1. Insulin unchanged, or increased (Johnson & Hazelwood, 1982; Rosebrough et al, 1984; Cherel et al, 1988),
2. Increased glucagon (Cieslak & Hazelwood, 1983; Hazelwood, 1986; Cherel et al, 1988),
3. Increased corticosterone during phase III of incubation fast, and during imposed short-term starvation (Cherel et al, 1988; Lea et al, 1992; Geris et al, 1999),
4. Plasma glucose unchanged during incubation or decreased during imposed starvation (Hazelwood & Lorenz, 1959; Harvey et al, 1978; Le Maho et al, 1981; Rosebrough et al, 1984),
6. Thyroxine decreased during incubation and either decreased, increased or unchanged during imposed starvation (Cherel et al, 1988; Groscolas & Leloup, 1989; Kühn et al, 1993; Schew et al, 1996; Darras et al, 1997; Prem et al, 1998; Geris et al, 1999; Van der Geyten et al, 1999),
7. Decreased thyroid stimulating hormone (Geris et al, 1999; Van der Geyten et al,

In both avian and mammalian species these changes in metabolic hormones act to maintain a constant internal environment. In mammals, decreased insulin concentrations decrease glucose use and promote the use of endogenous substrates for gluconeogenesis (Chilliard et al, 1998). Hypothyroidism decreases basal metabolism and reduces the rate of protein and fat turnover (Chilliard et al, 1998). The increased growth hormone concentration has a sparing effect on protein reserves (Norrelund et al, 2001). These hormonal changes minimize the loss of energy reserves.

A feature of starvation is the utilisation of adipose, and especially in birds, hepatic reserves (Kleiber, 1961; Hazelwood, 1986). The catabolism of these reserves releases substrates into the circulation that also alter metabolism. In mammalian species, acute starvation is associated with an increase in the circulating concentration of free fatty acids (Stout et al, 1976; Goschke & Tholen, 1977; Lyle et al, 1984; Romijn et al, 1991). In avian species a similar situation is observed during the first few days of fasting or incubation anorexia (Le Maho et al, 1981; Hazelwood, 1986). However, with prolonged incubation anorexia the concentration of free fatty acids decreases (Le Maho et al, 1981; Groscolas & Leloup, 1989). In humans, there is evidence that the elevation in free fatty acids in response to starvation suppresses gluconeogenesis and minimises protein degradation (Fery et al, 1996). As such, the response of animals to starvation provides insights into the control of appetite and the range of energy sparing mechanisms that are employed, when the animal's response are not influenced by the reproductive state.

Metabolite concentrations also alter in response to imposed or naturally occurring starvation. The changes in metabolite concentrations can reveal the energy source that the animal is using to meet its energetic requirements. In avian species, β-hydroxybutyrate rises rapidly in response to fasting, but at a slower rate in response to naturally occurring anorexia during incubation (Le Maho et al, 1981; Cherel et al, 1988). Increases in the concentration of β-hydroxybutyrate indicate catabolism of adipose stores. The later stages of incubation anorexia are associated with increased uric acid and decreased β-hydroxybutyrate plasma concentrations (Le Maho et al, 1981; Cherel et al, 1988; Groscolas & Leloup, 1989). This indicates that the birds are catabolising protein and obtaining less
energy from adipose reserves (Groscolas & Leloup, 1989). It is likely that the emu undergoes similar changes in metabolites, hormones and energy sparing mechanisms in response to starvation. As such, investigation of the emu's response to starvation could provide insights into the control of appetite and the nature of energy sparing mechanisms employed by the emu.

7.0 - Conclusions
The decreased appetite and diminished adipose reserves that are associated with the breeding season limit the commercial productivity of the emu. To overcome production constraints associated with reproduction in the emu it is essential that the physiology and biochemistry underpinning appetite in the emu be better understood. The information given in the preceding sections relates to a general integrated model for the control of energy balance that is based mainly on data from mammalian species. However, the emu may not adhere to this model given the strong evolutionary pressure applied to it for efficient use of available energy sources by the harshness of its environment.

The general aim of this study has been to improve the understanding of the control of appetite and adiposity in the emu. To address this aim, the general hypothesis of this thesis was that seasonal variation in appetite and adiposity in the emu operates under the general model developed in other species.

The general hypothesis was tested using experimental models associated with altered appetite, adiposity and metabolic hormone concentrations. The experimental models used were high fat diets and dexamethasone treatment (increased appetite and adiposity), incubation (decreased appetite and adiposity) and starvation (increased appetite and decreased adiposity). Three approaches were taken to test the general hypothesis. First, to determine the role of leptin in the control of appetite and adiposity attempts were made to clone and sequence the emu leptin gene. Once sequenced it was intended that the gene would be used to develop an emu specific leptin radioimmunoassay, and investigate changes in leptin gene expression during the annual cycle of the emu. Second, the concentration of metabolic hormones was measured in all of the experiments. Finally, to determine the role of NPY in the control of appetite and adiposity the mRNA expression of NPY from the mediobasal hypothalamus and preoptic area was quantified in the glucocorticoid treatment, incubation and starvation experiments.
Chapter 2

General Materials and Methods

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10. Molecular techniques 52
A general overview of the materials and methods employed during the course of the study is presented here. Details of the techniques, treatments and analyses will be given fully in the experimental chapters. All experiments were approved by the University of Western Australia animal ethics committee and were carried out in accordance with welfare guidelines. Appendix 2 contains a list of all suppliers mentioned in this and subsequent chapters.

1. Experimental Location
Experiments were performed at the Shenton Park research station (31°56'S), part of the University of Western Australia, situated in Perth, Western Australia. Perth has a mediterranean climate, with wet winters (average rainfall 150.9 mm; daily temperature range 8.1-28.3°C) and hot, dry summers (average rainfall 12 mm; daily temperature range 14.8-46.7°C) (Bureau of Meterology website). All animals used in the experiments were maintained outdoors in individual pens (pen size: experiments 1, 3 and 4 were greater than 15 m², experiment 2 were approximately 4 m²).

2. Animals
All emus used during the course of this research were either bred at the Shenton Park facility (UWA), Medina (Agriculture Western Australia) or obtained from a commercial breeder in the vicinity of Perth (P & T Amrein, Wungong). The animals were acclimatised at the Shenton Park facility in individual pens for at least two weeks before the commencement of any experiments. The animals used were sexually mature adults, ranging in age from 14 months to five years old, unless otherwise stated. Due to a downturn in the emu market birds were not readily available and therefore the choice of sex for each experiment was determined by availability.

3. Nutrition
Animals were fed an emu breeder pellet obtained from Glen Forrest Stockfeeders (for specifications see Appendix 2). Feed and water were provided *ad libitum* for all experiments, unless otherwise stated.

4. Measurements
*Live weight*
Animals were weighed periodically during the course of all the experiments. The birds were caught, manoeuvred onto livestock scales (Ruddweigh KM-2 electronic weighing system), and held while recordings were made (Fig. 2.1).

![Figure 2.1: Live weight measurement of the emu.](image)

**Fat score**

An assessment of the size of the subcutaneous fat depot, as developed by Mincham *et al* (1998), was conducted periodically for experiment 2. This entailed restraining the bird while the region between the preacetabular ilium and postacetabular ilium was palpatated. The body condition of the animal in terms of build up of muscle and fat around the backbone was scored between 1 and 5. The values correspond to the following descriptions:

1 – bones prominent and sharp;
2 – bones prominent but smooth;
3 – bones can be felt but are smooth and rounded;
4 – bones are detectable with pressure on the thumb;
Feed Intake
Throughout the experiments individual feed intakes were recorded. Buckets were filled with feed such that the combined weight was in the range of 6 to 8 kilograms. The exact weight was recorded and the buckets returned to the pens. After periods of either 48 or 72 hours the buckets were collected and the combined weight of the bucket and the remaining feed was recorded. The buckets were then refilled, weighed, recorded and returned to the bird. Weighing and refilling of buckets was done in the morning.

Blood glucose
Blood glucose was measured during experiments’ 2 and 3, using a hand-held blood glucose meter (Precision Q.I.D, Medisense) by applying a droplet of blood to an electrode (Precision plus, Medisense) immediately following each bleed. The blood glucose levels recorded for emus ranged from 5.1 to 16.5 mmol/L. The between electrode variation determined by the manufacturer is 2.1-3.7% over the range of concentrations observed for emus. Parallelism had been previously determined for emus (Van Cleeoff, 2001).

Carcass composition
Following the removal of tissue samples (further details are given in the tissue collection techniques below), the carcass was stripped of subcutaneous adipose tissue. Following this the internal organs were removed and the visceral fat dissected out. The tissues of interest were then weighed with the whole procedure taking approximately 45 minutes per bird (Table 2.1). The digestive tract length (proventriculus to cloaca) was also measured for experiment 1 (Chapter 4).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous adipose</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Visceral adipose</td>
<td>✓</td>
<td>✓</td>
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<td>✓</td>
</tr>
<tr>
<td>Liver</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Gastrocnemius lateralis (hamstring)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td></td>
<td>✓</td>
<td></td>
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<tr>
<td>Testis</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Digestive tract (proventriculus to cloaca)</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experiments 1, 2, 3 and 4 are described in Chapters 4, 5, 6 and 7 respectively.
Tissue fat content determination

Following slaughter the fat content of liver and muscle was determined for experiment 1 using the method developed within the Animal Science group and described by Pluske (1993). Approximately 2 g (wet weight) samples of each tissue were collected in triplicate. Samples were frozen at -20°C, freeze-dried and re-weighed. The tissues were then ground to a powder and mixed with 40 ml of solvent (hexane:isopropanol = 3:2), and tumbled at a slow speed for two hours before being left to stand overnight. The samples were filtered under vacuum through Whatman no.1 filter paper over a ceramic filter with 3 micron pores. The filtrate was dried for two hours at 70°C to evaporate excess solvent and weighed. The fat content was determined as the amount removed by the solvent:

\[
\text{fat content} = \text{freeze-dried weight} - \text{weight of tissue after solvent extraction.}
\]

Figure 2.2: Jugular bleeding of the emu.

5. Blood sampling

The animals were caught and restrained by holding their wings while standing behind them. This was necessary to control the animal while being bled. Blood samples were taken at regular intervals from the jugular using an 18-gauge needle and either a 10 or 20 ml syringe (Fig. 2.2). Between 10-20 ml samples were collected and immediately transferred into plastic tubes for plasma or serum collection. Plasma collection tubes contained 40 IU/ml of heparin with a small amount of polystyrene granules (Kwikspin granules: Disposable Products Pty Ltd, Australia) added. Plasma tubes were centrifuged...
(Sarstedt Australia Pty Ltd) and were stored at 4°C for 1-3 days before being centrifuged. All centrifugation of blood samples was performed at 3000 rpm and 4°C for 25 minutes. Plasma and serum samples were decanted into plastic tubes (Sarstedt Australia Pty Ltd) and stored at -20°C. All blood samples were collected in the morning.

6. Euthanasia:
All birds were euthanased at the completion of experiments by the injection of 25 ml of Lethabarb (sodium pentabarbitone 325 mg/ml, Virbac) into the jugular.

7. Tissue collection techniques for:

RNA extraction
Samples were collected for RNA extraction from the following tissues: adipose, liver, mediobasal hypothalamus and preoptic area. To obtain the mediobasal hypothalamus and preoptic area the head was removed following euthanasia and the skull opened using secateurs to cut through the skull in a hexagonal pattern (Fig. 2.3). The piece of skull bounded by the cuts was then pried up along the incision at the back of the skull, and the dura mere carefully cut away to remove it without disturbing the brain. Incisions were made through the cerebellum and the optic chiasma, and the brain gently lifted free by cutting through any constraining connective tissue and nerves. The region of the brain containing the mediobasal hypothalamus and the preoptic area were collected and snap frozen in liquid nitrogen for RNA extraction (Fig. 2.4). All tissues required were excised within 15 minutes of the animal being euthanased and cut into appropriately sized pieces (not greater than one gram). To prevent RNA degradation by RNases prior to tissue collection all instruments used were wrapped in alfoil and baked at greater than 200°C for 24 hours, and any manipulation of tissues was carried out either on baked alfoil or in baked glass petri dishes. Where instruments were re-used they were rinsed in a 2% SDS solution between animals. Sterile 5 ml tubes (Sarstedt Australia Pty Ltd) were used to store tissues and samples were snap frozen in liquid nitrogen as rapidly as possible after collection (less than one minute). Samples were stored at -80°C until RNA extractions were performed (section 10.).
Figure 2.3: An emu skull showing the placement of incisions through the skull for brain removal.

Figure 2.4: Schematic sagittal view of the avian brain showing the microdissection sites for collection of the mediobasal hypothalamus (MBH) and preoptic area (POA) for RNA extraction and RPA (CER=cerebellum, PS=pituitary stalk, OC=optic chiasma). Adapted from Pearson (1972).
8. Metabolic substrate and hormone assays

General buffers and reagents

0.5 M phosphate buffer
To ~800 ml of nanopure water add 61.3 g disodium hydrogen orthophosphate (Na$_2$HPO$_4$) and 10 g sodium dihydrogen orthophosphate (NaH$_2$PO$_4$·2H$_2$O), adjust the pH to 7.5 and bring the volume to 1 L with nanopure water.

0.3 M phosphate buffer
3:2 dilution of 0.5 M phosphate buffer in nanopure water, pH 7.5.

0.1 M phosphate buffer
1:4 dilution of 0.5 M phosphate buffer in nanopure water, pH 7.5.

Phosphate buffered saline (PBS)
100 ml 0.1 M phosphate buffer, 8.9 g sodium chloride (NaCl), 1 g sodium azide (NaN$_3$) make to 1 L with nanopure water, pH 7.5.

Sample preparation
Emu serum and plasma often exhibit high lipid contents. The lipid interferes with the detection of luteinising hormone (LH), glucagon and insulin via radioimmunoassay. To remove the lipid two approaches were employed. Samples to be assayed for LH and glucagon (1 ml) were treated with 45% polyethylene glycol (PEG) (200 μl), incubated for 30 minutes and centrifuged at 1600 g for 10 minutes (Blache et al, 2001a). The clear plasma or serum was transferred to a fresh tube. PEG extraction recovers 75%, 89% and 40% respectively of LH, glucagon and insulin (Van Cleeff, 2001). As the recovery of insulin by PEG extraction is low, lipid removal from samples to be assayed for insulin was achieved by centrifugation at high speed for between 10 and 30 minutes. The clear plasma or serum was removed from below the fat layer to a fresh tube.

Corticosterone
Corticosterone was measured using extraction methods developed by Smith et al (1972) and Etches (1976).

Buffers/reagents

Gelatin phosphate buffer (GPB)  
PBS (pH 7.5) + 1 g gelatin/L

Dextran-coated charcoal  
6.25 g charcoal + 0.625 g dextran T70/L in GPB.
Standards

A stock solution of corticosterone (4-pregnene-11β, 21-diol-3, 20-dione, Sigma) was prepared in ethanol to a concentration of 7.6 μg/ml. Standards were made by serial dilution to the following concentrations: 20, 10, 5, 2.5, 1.25, 0.6125, 0.306, 0.15 and 0.078 ng/μl in ethanol.

Antiserum

The first antibody, corticosterone antiserum B3-163, was raised in rabbits and obtained from Endocrine Sciences Products. To use in the assay it was reconstituted, and diluted to 1:200 in gelatin phosphate buffer.

Tracer

[1,2,6,7-3H] corticosterone (Amersham Biosciences Pty Ltd).

Purification:

A 5 ml pipette stoppered with a glass bead was filled with 2.5 ml of LH-20 sephadex (Pharmacia Biotech) swollen in a mixture of benzene:methanol (9:1). 10 μCi of tracer was dried down and redissolved in benzene:methanol (9:1) (100 μl). This was added to the column and allowed to enter the gel bed. The column was eluted with benzene:methanol (9:1). Fractions (500 μl) were collected into glass tubes and an aliquot of 5 μl of each fraction was counted with scintillant (Starscint, Packard) using a liquid scintillation counter (Packard 1500 Tri-Carb Liquid Scintillation Analyzer). The appropriate fractions were pooled, dried down under compressed air and dissolved in absolute ethanol (5 ml). On Day 1 of the assay the tracer was diluted to 10000 cpm per 20 μl.

Assay procedure

The standard curve was placed at the beginning of the assay. Each standard curve included 3 tubes for total counts (TC), 3 tubes for non-specific binding (NSB), 9 replicates of zero binding (B0), 3 replicates of each standard and 4 replicates each of two pool samples.

On Day 1, the tubes to be used for the assay were coated with 10000 cpm of the tracer prior to commencement of the assay. 12 x 75 mm glass tubes were coated for samples and pools and 10 x 75 mm glass tubes were coated for standards, TC, NSB and B0. Tracer (20 μl, 10000 cpm) was added to all tubes, standards (100 μl) were added in
triplicate to the appropriate tubes. All tubes were evaporated to dryness under compressed air at 37°C using a heated block.

Extraction:
Samples of unknown serum and pools (100 μl) were added to the coated tubes, vortexed individually and incubated for 30 minutes at room temperature. The samples and pools were extracted by adding 2 ml of 2,2,4-trimethyl pentane (iso-octane) or hexane to each tube and vortexing twice for 30 seconds. The tubes were then centrifuged at 1500 rpm for 5 minutes (Beckman J-6M/E centrifuge). The aqueous layer was frozen by partially immersing the tubes in an acetone-dry ice bath, the solvent layer was then discarded. Dichloromethane (2 ml) was added to each tube and the tubes were vortexed twice for 30 seconds. The tubes were centrifuged at 1500 rpm for 5 minutes (Beckman J-6M/E centrifuge). The tubes were returned to the acetone-dry ice bath to freeze the upper aqueous layer, the organic layer was then poured off into clean 10 x 75 mm glass tubes. The dichloromethane was evaporated to dryness under compressed air at 37°C. First antibody (250 μl) was added to all tubes except the TC and NSB (GPB (250 μl) was added to TC and NSB in place of the first antibody). The tubes were then shaken and incubated for 24 hrs at 4°C.

On Day 2, dextran-coated charcoal (200 μl) was added to all tubes except TC the tubes were incubated for 20 minutes at 4°C before being centrifuged at 1500 rpm for 10 minutes (Beckman J-6M/E centrifuge). A volume of 400 μl of the supernatant was then removed and added to a counting vial along with 2 ml of scintillant (Starscint, Packard) and counted for 3 minutes each in a liquid scintillation counter (Packard 1500 Tri-Carb Liquid Scintillation Analyzer).

Glucagon
Glucagon was measured using Linco kit tracer, standards and first antibody (Linco Research, Inc.). The assay was validated for emus by Van Cleeff (2001).

Buffers/reagents

**Glycine buffer**

15 g glycine, 10 g bovine serum albumin (BSA), 9 g ethylenediaminetetra acetic acid (EDTA) (non-salt), 1 g NaN₃. Dissolve in 800 ml of nanopure
water, adjust pH to 8.8 and bring volume to 1 L with nanopure water.

**Tracer buffer**

0.375 g glycine, 2.5 g BSA, 2.25 g EDTA (non-salt), 0.2 g NaN₃. Dissolve in 150 ml of nanopure water, adjust pH to 8.8 and bring volume to 250 ml with nanopure water.

**Standards**

Purified recombinant human glucagon (800 pg/ml) was used to make the standards by serial dilution to the following concentrations: 400, 200, 100, 50, 25 and 12.5 pg/ml in glycine buffer.

**Antiserum**

First antibody:

Guinea pig anti-glucagon, specific for pancreatic glucagon. Reconstituted on arrival with 100 ml of glycine buffer and 100 µg of aprotinin.

Second antibody:

Sheep anti-guinea pig raised in the laboratory and diluted 1:20 in glycine buffer with normal guinea pig serum (NGPS) added at a dilution of 1:400.

**Tracer**

¹²⁵I-glucagon obtained from Linco. Reconstituted by adding 27 ml of tracer buffer per vial, and occasional gentle mixing for 30 minutes. This was further diluted to >12,000 counts per 100 µl.

**Assay procedure**

The standard curve was placed at the beginning of the assay. Each standard curve included 3 tubes for TC, 3 tubes for NSB, 9 replicates of B₀, 3 replicates of each standard and 4 replicates each of three pool samples.

On Day 1, unknown plasma samples (50 µl), and pools and standards (100 µl each) were diluted to a final volume of 200 µl with glycine buffer in 12 x 75 mm plastic tubes (Sarstedt Australia). Glycine buffer was added to NSB (300 µl) and B₀ (200 µl) tubes. The first antibody preparation (100 µl) was added to all tubes except the NSB and TC. Tubes were then incubated overnight at 4°C.
On Day 2, tracer (100 µl) was added to all tubes and mixed. The tubes were incubated overnight at 4°C.

On Day 3, second antibody (100 µl) was added to all tubes except the TC, mixed and incubated overnight at 4°C.

On Day 4, 3% PEG in PBS (pH 8.8) (1 ml) was added to all tubes except the TC, before centrifugation at 3000 rpm for 25 minutes at 4°C (Beckman J-6M/E centrifuge) after which the supernatant was poured off. The TC were not centrifuged or poured off. The activity of the precipitate was counted for 90 seconds each in a gamma counter (Packard Cobra II Auto-Gamma).

Insulin
Insulin was measured in plasma or serum using the method for chickens developed by McMurtry et al (1983). This method was validated for emu plasma and serum by Van Cleeoff (2001). The chicken insulin used for the standards and tracer, and the first antibody were obtained from Professor McMurtry, USDA.

Buffers/reagents

<table>
<thead>
<tr>
<th>Buffer/Reagent</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin buffer</td>
<td>1.095 g NaH₂PO₄.2H₂O, 5.906 g Na₂HPO₄, 18.6125 g EDTA (disodium salt). Dissolve in 800 ml of nanopure water, adjust pH to 7.5, and bring volume to 1 L.</td>
</tr>
<tr>
<td>Chloramine-T 0.3 M phosphate</td>
<td>5 mg chloramine-T in 10 ml 0.3 M phosphate buffer, prepared 10 minutes before the iodination.</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>5 mg sodium metabisulphite (Na₂S₂O₅) in 10 ml 0.3 M phosphate buffer.</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>200 mg potassium iodide (KI) in 10 ml 0.1 M phosphate buffer.</td>
</tr>
</tbody>
</table>

Standards
Standards were made by serial dilution of a 10 ng/ml stock of chicken insulin to the following concentrations: 7.5, 5, 3.75, 2.5, 1.875, 1.25, 0.9375, 0.625, 0.46875, 0.3125, 0.234 and 0.15625 ng/ml in insulin buffer + 0.1% gelatine.
Antiserum
First antibody:
The first antibody was raised in guinea pigs against chicken insulin. It was used at 1:15,000 with 1:600 of normal guinea pig serum diluted in insulin buffer + 0.1% gelatine.
Second antibody:
Sheep anti-guinea pig, raised in the laboratory and diluted 1:10 with insulin buffer.

Tracer
Columns:
G100 (Pharmacia) equilibrated with 1% BSA, 0.1 M phosphate buffer
Iodination:
Chicken insulin was stored as 5 µg aliquots at -80°C. To a 5 µg aliquot, 0.5 mCi of $^{125}$I and chloramine-T buffer (10 µl) was added, mixed and allowed to incubate at room temperature for 90 seconds. Potassium iodide (100 µl) and sodium metabisulphite (100 µl) solutions were added to stop the reaction. The reaction was added to the column, and the reaction vessel rinsed with 1% BSA, 0.1 M phosphate buffer (100 µl). The rinse solution was also added to the column and 1 ml fractions were eluted with 1% BSA, 0.1 M phosphate buffer. An aliquot of 10 µl of each fraction was counted for 10 seconds in a gamma counter (Packard Cobra II Auto-Gamma) to identify the appropriate fractions. The tracer was immediately diluted to >15,000 counts/100 µl with insulin buffer + 1% BSA and added to the assay. Excess tracer was stored at 4°C.

Assay procedure
The standard curve was placed at the beginning of the assay. Each standard curve included 3 tubes for TC, 4 tubes for NSB, 9 replicates of $B_0$, 3 replicates of each standard and 4 replicates each of two pool samples.

On Day 1, unknown plasma samples and pools (100 µl) were diluted to 200 µl with insulin buffer + 0.1% BSA (100 µl) in 12 x 75 mm plastic tubes (Sarstedt Australia). Standards (200 µl) were added to appropriate tubes. Insulin buffer + 0.1% BSA was added to NSB (300 µl) and $B_0$ (200 µl). The first antibody preparation (100 µl) was added to all tubes except the NSB and TC. Tubes were then incubated overnight at 4°C.
On Day 2, tracer (100 μl) was added to all tubes and mixed. The tubes were incubated for 24 hours at 4°C.

On Day 3, second antibody (100 μl) was added to all tubes except the TC, mixed and incubated overnight at 4°C.

On Day 4, 6% PEG in PBS (1 ml) was added to all tubes except the TC, before centrifugation at 3000 rpm for 25 minutes at 4°C (Beckman J-6M/E centrifuge) after which the supernatant was poured off. The TC were not centrifuged or poured off. The activity of the precipitate was counted for 1 minute each in a gamma counter (Packard Cobra II Auto-Gamma).

Luteinising hormone (LH)
LH was measured in plasma using the method described by Talbot et al (1988), based on the original LH assay developed by Follet et al (1972). The validation of this method for emu plasma is described by Malecki et al (1998) and Blache et al (2001a). The chicken LH fraction (PRC-AEI-s-1) used for the assay standards and iodination was kindly donated by Professor Sharp, Roslin Institute, Midlothian, Scotland. This was prepared using the method described by Talbot et al (1988). The fraction was purified by passage through a G100 column (Pharmacia Biotech) and had a potency of 0.43 (0.375-0.499) NIH-o-LH-S19 in the chicken granulosa cell assay (Hammond et al, 1980).

Buffers/reagents

**LH buffer**
80 ml 0.5 M phosphate buffer, 8.75 g NaCl, 10 mM EDTA, 1 g NaN₃, 1 g BSA, bring to 900 ml with nanopure water, adjust pH to 7 with 1 N sodium hydroxide (NaOH), and bring to 1 L with nanopure water.

**0.1 M phosphate-gelatine**
0.1 M phosphate buffer, 0.25% gelatine in nanopure water, pH 7.5.

**0.1 M phosphate-BSA**
0.1 M phosphate buffer, 1% BSA.

**Chloramine-T 0.1 M phosphate**
5mg chloramine-T in 10 ml 0.1 M phosphate buffer, prepared 10 minutes before the iodination.

**Sodium metabisulphite**
10 mg Na₂S₂O₅ in 100 ml 0.1 M phosphate buffer.
**Potassium iodide**  
1 g KI in 10 ml 0.1 M phosphate buffer.

**Standards**
Aliquots of 1.25 µg of LH in nanopure water were stored at -20°C. The LH solution was diluted with LH buffer to give a 250 ng/ml working stock solution. Standards were made by serial dilution to the following concentrations: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 ng/ml in LH buffer.

**Antiserum**
First antibody:
Raised in a rabbit immunised against the chicken LH preparation supplied by Professor Sharp. The stock (1:200 dilution) was diluted further with LH buffer to give a working dilution of 1:20,000.

Second antibody:
Donkey anti-rabbit raised in the laboratory. The stock was diluted with LH buffer (1:6) to give the working solution.

**Tracer**
Columns:
G25 column (Pharmacia) equilibrated with 0.1 M phosphate-gelatine buffer + 2% BSA.

Iodination:
To 1.25 µg LH in 10 µl nanopure water, 0.5 mCi Na-125I and chloramine-T buffer (20 µl) were added and incubated for 60 seconds. The reaction was stopped by adding sodium metabisulphite (100 µl) and potassium iodide (200 µl). The column was drained down to the gel surface. The contents of the reaction vessel were then placed on the column. The reaction vessel was rinsed with potassium iodide buffer (250 µl) that was then added to the column. Fractions (500 µl) were eluted with 0.1 M phosphate-gelatine buffer into tubes containing 500 µl 0.1 M phosphate-BSA buffer. On Day 2 of the assay the appropriate fraction was diluted to 10,000 counts per 50 µl with LH buffer.

**Assay procedure**
The standard curve was placed at the beginning of the assay. Each standard curve included 3 tubes for TC, 3 tubes for NSB, 9 replicates of B₀, 3 replicates of each standard and 4 replicates each of two pool samples.
On Day 1, unknown plasma samples and pools (50 μl) were diluted to 200 μl with LH buffer in 12 x 75 mm plastic tubes (Sarstedt Australia). Standards (200 μl) were added to the appropriate tubes. LH buffer was added to NSB (250 μl) and B0 (200 μl) tubes. The first antibody (50 μl) was added to all tubes except the NSB and TC. Tubes were then incubated overnight at 4°C.

On Day 2, tracer (50 μl) was added to all tubes and vortexed briefly. The tubes were incubated for 48 hours at 4°C.

On Day 4, NRS (1:400 dilution with LH buffer) (50 μl) and second antibody (50 μl) were added to all tubes except the TC, vortexed briefly and incubated overnight at 4°C.

On Day 5, 6% PEG in LH buffer (1 ml) was added to all tubes except the TC, before centrifugation at 1500 g for 30 minutes at 4°C (Beckman J-6M/E centrifuge) after which the supernatant was poured off. The TC were not centrifuged or poured off. The activity of the precipitate was then counted for 1 minute each in a gamma counter (Packard Cobra II Auto-Gamma).

Prolactin

Prolactin was measured in plasma using the method developed by Talbot and Sharp (1994). Validation of this method for emu prolactin is described by Malecki et al (1998). Recombinant derived chicken prolactin used for the assay standards, raising the first antibody and iodination was kindly donated by Professor Sharp, Roslin Institute, Midlothian, Scotland. Recombinant chicken prolactin was prepared using the method described by Hanks et al (1989).

Buffers/reagents

<table>
<thead>
<tr>
<th>Buffer/reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramine-T 0.3 M phosphate</td>
<td>5 mg chloramine-T in 10 ml 0.3 M phosphate buffer.</td>
</tr>
<tr>
<td>2% BSA</td>
<td>0.2 g BSA in 10 ml 0.3 M phosphate buffer.</td>
</tr>
<tr>
<td>Tris/Tween 20 (make fresh)</td>
<td>1.8 g NaCl, 3.14 g tris/HCl, 0.1 g NaN₃, 200 μl Tween 20, bring to 200 mL in nanopure water, adjust pH to 8.</td>
</tr>
<tr>
<td>Prolactin buffer</td>
<td>900 ml 0.1 M phosphate buffer (pH7.5), 9 g NaCl, 3.72 g EDTA (disodium salt), 1 g NaN₃, 1 g BSA.</td>
</tr>
</tbody>
</table>
Adjust pH to 7 with 1 N NaOH and bring to 1 L with nanopure water.

**Standards**

The prolactin stock was diluted with prolactin buffer to give a 1250 ng/ml working stock solution. Standards were made by serial dilution to the following concentrations: 125, 62.5, 31.25, 15.625, 7.8125, 3.9, 1.95, 0.977, 0.4883 and 0.24 ng/ml in prolactin buffer.

**Antiserum**

First antibody:
Raised in a rabbit immunised against recombinant chicken prolactin by Professor Sharp. The stock (1:100 dilution) was diluted further with prolactin buffer to give a 1:6000 working solution.

Second antibody:
Donkey anti-rabbit raised in the laboratory. The stock solution (1:2 dilution) was further diluted with prolactin buffer (1:1) to give the working solution.

**Tracer**

Columns:
PD10 (Pharmacia Biotech) and G100 (Pharmacia Biotech) equilibrated with Tris/Tween 20 buffer.

Iodination:
Prolactin stock (14 μl, 5 μg) was aliquoted into an eppendorf tube and 0.3 M phosphate buffer (16 μl), $^{125}$I-Na (5 μl) and chloramine-T buffer (10 μl) were added. The reaction vessel was vortexed and incubated at room temperature for 60 seconds. The reaction was stopped by the addition of 0.3 M phosphate buffer (200 μl). The reaction solution was applied to a PD 10 column and the reaction tube rinsed with 0.3 M phosphate buffer (250 μl) the rinse product was also applied to the column. Fresh Tris/Tween 20 buffer was used to elute 11-drop fractions. The appropriate fractions were identified by counting 10 μl aliquots using a gamma counter (Packard Cobra II Auto-Gamma), and then combined. The G 100 column was allowed to drain down to the gel surface and the iodinated fraction applied. Fractions (1 ml) were eluted with Tris/Tween 20 buffer. The appropriate fraction was identified by counting 10 μl aliquots using a gamma counter.
The fraction was diluted 1:1 with 2% BSA. On Day 2, the appropriate fraction was diluted to 12,000 counts per 50 μl with prolactin buffer.

**Assay procedure**

The standard curve was placed at the beginning of the assay. Each standard curve included 3 tubes for TC, 3 tubes for NSB, 9 replicates of B₀, 3 replicates of each standard and 4 replicates each of three pool samples.

On Day 1, unknown plasma samples and pools (100 μl) were added to 12 x 75 mm plastic tubes (Sarstedt Australia). The standards (100 μl) were added to appropriate tubes. The first antibody (50 μl) was added to all tubes except the NSB and TC. Prolactin buffer (150 μl and 100 μl respectively) was added to NSB and B₀ tubes. Tubes were incubated overnight at 4°C.

On Day 2, tracer (> 12000 counts/50 μl) (50 μl) was added to all tubes and vortexed briefly. The tubes were incubated for 24 hours at 4°C.

On Day 3, NRS (1:600 dilution with prolactin buffer) (50 μl) and second antibody (50 μl) were added to all tubes except the TC, vortexed briefly and incubated overnight at 4°C.

On Day 4, 6% PEG in PBS (1 ml) was added to all tubes except the TC, before centrifugation at 1500 g for 30 minutes at 4°C (Beckman J-6M/E centrifuge) after which the supernatant was poured off. The TC were not centrifuged or poured off. The activity of the precipitate was then counted for 1 minute each in a gamma counter (Packard Cobra II Auto-Gamma).

**Thyroid Hormones**

Total triiodothyronine (T3) and total thyroxine (T4) levels in serum and plasma were measured using a non-extraction assay described by Dawson *et al* (1996) and Dawson and Deeming (1997). The validation of this method for emu plasma is described by Blache *et al* (2001c).
Buffers/reagents

Barbitol buffer
12.4 g barbital sodium (C₈H₁₁N₂NaO₃) make up to 1 L in nanopure water, pH 8.6.

ANSA buffer
0.8 g 8-anilino-1-naphthalene sulphonic acid (ANSA) to 100 ml of barbitol buffer.

T3 standards
A stock solution of T3 was prepared by dissolving 10 mg of T3 powder in 100 ml of barbitol buffer. It was further diluted 1:1000 to give a final concentration of 100 ng/ml. Standards were made by serial dilution to the following concentrations: 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.0975, 0.049, 0.0245, 0.012 and 0.006 ng/ml in barbitol buffer.

T3 antiserum
First antibody:
Rabbit polyclonal antibody anti-T3 was purchased from Biogenesis Immuno Diagnostics, and diluted 1:10 on arrival in barbitol buffer. For the assay it was diluted 1:40 to give a 1:400 stock. The first antibody was added to the assay at a working dilution of 1:12,000.
Second antibody:
Donkey anti-rabbit raised in the laboratory and diluted 1:5 with barbitol buffer.

T3 tracer
L-3,5,3'-[¹²⁵I]- triiodothyronine (NEN Life Science Products, Inc.) diluted in ANSA buffer to give 10,000 cpm/20 µl

T3 assay procedure
The standard curve was placed at the beginning of the assay. Each standard curve included 3 tubes for TC, 3 tubes for NSB, 9 replicates of B₀, 3 replicates of each standard and 3 replicates each of three pool samples.
On Day 1, standards (20 µl) were diluted to a total volume of 60 µl, with charcoal stripped plasma (20 µl) and barbitol buffer (20 µl). The unknown serum samples and pools (20 µl) were diluted to a total volume of 60 µl with barbitol buffer (40 µl). 10 x 75 mm glass tubes were used (Sarstedt Australia). The first antibody (20 µl) was added
to all tubes except the NSB and TC. Tracer (20 μl) was added to all tubes and vortexed briefly. The tubes were incubated for 48 hours at 4°C.

On Day 3, second antibody and normal rabbit serum (NRS) (1:500 dilution) (20 μl each) were added to all tubes except the TC, and vortexed briefly. The tubes were then incubated for 24 hours at 4°C.

On Day 4, 6% PEG in barbitol buffer (1 ml) was added to all tubes except the TC, before centrifugation at 1500 g for 25 minutes at 4°C (Beckman J-6M/E centrifuge) after which the supernatant was aspirated. The TC were not centrifuged or aspirated. The activity of the precipitate was then counted for 1.5 minutes each in a gamma counter (Packard Cobra II Auto-Gamma).

**T4 standards**

A working stock solution of T4 was made by adding 100 μl of a 12.8 μmol/L concentrated stock solution to 10 ml of barbitol buffer. Standards were made by serial dilution to the following concentrations: 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 nM/L in barbitol buffer.

**T4 antiserum**

First antibody:

Rabbit polyclonal antibody anti-T4 was purchased from Biogenesis Immuno Diagnostics, and diluted 1:10 on arrival in barbitol buffer. For the assay it was diluted 1:40 to give a 1:400 stock. The first antibody was added to the assay at a working dilution of 1 in 6,400.

Second antibody:

Donkey anti rabbit raised in the laboratory and diluted 1:5 with barbitol buffer.

**T4 tracer**

L-[¹²³I]-thyroxine (NEN Life Science Products, Inc.) diluted in ANSA buffer to give 10,000 cpm/20 μl.

**T4 assay procedure**

The standard curve was placed at the beginning of the assay. Each standard curve included 3 tubes for TC, 3 tubes for NSB, 9 replicates of B₀, 3 replicates of each standard and 3 replicates each of three pool samples.
On Day 1, standards (20 µl) were diluted to a total volume of 60 µl, with charcoal stripped plasma and barbitol buffer (20 µl each). The unknown serum samples and pools (20 µl) were diluted to a total volume of 60 µl with barbitol buffer (40 µl). 10 x 75 mm glass tubes were used (Sarstedt Australia). The first antibody (20 µl) was added to all tubes except the NSB and TC. Tracer (20 µl) was added to all tubes and vortexed briefly. The tubes were incubated for 24 hours at 4°C.

On Day 2, second antibody and NRS (1:500 dilution) (20 µl each) were added to all tubes except the TC, and vortexed briefly. The tubes were then incubated for 24 hours at 4°C.

On Day 3, 6% PEG in barbitol buffer (1 ml) was added to all tubes except the TC, before centrifugation at 1500 g for 25 minutes at 4°C (Beckman J-6M/E centrifuge) after which the supernatant was aspirated. The TC were not centrifuged or aspirated. The activity of the precipitate was then counted for 1.5 minutes each in a gamma counter (Packard Cobra II Auto-Gamma).

9. Hormone data analysis

Calculation of hormone assay results

The final counts obtained for each assay were saved onto MS-DOS disks and converted into Macintosh format. Concentrations of the hormones were then calculated using the AssayZap Universal Assay Calculator.

Sensitivity and precision

The NSB, B₀, and assay sensitivity for each hormone assay are summarised in Table 2.2. The sensitivity of the assay is defined as the limit of detection of the standard curve. The precision of the assay expressed as intra-assay and inter-assay coefficients of variation are given in Table 2.3. For each hormone assayed, the intra-assay coefficient of variation was calculated from the standard deviation among replicates of each quality control sample. In Tables 2.2 and 2.3, the assay statistics and intra-assay coefficients of variation for glucagon, T3, T4 and corticosterone are presented separately for the assays performed, because the hormone concentrations from the samples were measured within one assay for each experiment.
Pools for assessing intra-assay coefficients of variation

Whenever possible, plasma pools were prepared for high, medium and low concentrations of each hormone.
Table 2.2: Assay statistics.

<table>
<thead>
<tr>
<th>Assay</th>
<th>NSB%</th>
<th>B₀%</th>
<th>Detection limit</th>
<th>High pool</th>
<th>Medium pool</th>
<th>Low pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL ng/ml (1)</td>
<td>3.1</td>
<td>42.3</td>
<td>0.24</td>
<td>5.29±0.39</td>
<td>1.40±0.05</td>
<td>1.02±0.08</td>
</tr>
<tr>
<td>T₃ ng/ml (3) *</td>
<td>5.2</td>
<td>22.1</td>
<td>0.11</td>
<td>1.10±0.189</td>
<td>-</td>
<td>0.06±0.003</td>
</tr>
<tr>
<td>T₄ ng/ml (1)</td>
<td>3.7</td>
<td>28.3</td>
<td>0.05</td>
<td>1.38±0.137</td>
<td>0.65±0.054</td>
<td>0.44±0.081</td>
</tr>
<tr>
<td>CORT ng/ml (4) *</td>
<td>6.1</td>
<td>24.8</td>
<td>0.03</td>
<td>0.60±0.029</td>
<td>0.40±0.040</td>
<td>0.16±0.030</td>
</tr>
<tr>
<td>PRL ng/ml (1)</td>
<td>4.1</td>
<td>19.9</td>
<td>0.60</td>
<td>3.03±0.052</td>
<td>2.18±0.211</td>
<td>0.36±0.045</td>
</tr>
<tr>
<td>T₃ ng/ml (3) *</td>
<td>2.6</td>
<td>40.5</td>
<td>0.30</td>
<td>5.23±0.426</td>
<td>3.45±0.058</td>
<td>2.76±0.159</td>
</tr>
<tr>
<td>T₄ ng/ml (1)</td>
<td>4.1</td>
<td>40.0</td>
<td>0.16</td>
<td>4.27±0.033</td>
<td>-</td>
<td>1.10±0.173</td>
</tr>
<tr>
<td>CORT ng/ml (4) *</td>
<td>3.6</td>
<td>40.9</td>
<td>0.18</td>
<td>9.43±0.329</td>
<td>-</td>
<td>1.98±0.184</td>
</tr>
<tr>
<td>GLUC pg/ml (2) *</td>
<td>1.7</td>
<td>26.4</td>
<td>0.16</td>
<td>5.06±0.535</td>
<td>-</td>
<td>0.24±0.023</td>
</tr>
<tr>
<td>INS ng/ml (1)</td>
<td>8.1</td>
<td>32.3</td>
<td>0.33</td>
<td>0.81±0.024</td>
<td>0.65±0.028</td>
<td>0.44±0.036</td>
</tr>
</tbody>
</table>
| PRL = prolactin, T₃ = triiodothyronine, T₄ = thyroxine, CORT = corticosterone, INS = insulin, GLUC = glucagon, LH = luteinising hormone. The number of assays performed for each hormone is given in brackets. An * indicates that different pools were used for all the assays performed.

Table 2.3: The intra-assay coefficients of variation for the hormone assays performed.

<table>
<thead>
<tr>
<th>Assay</th>
<th>High Pool (%)</th>
<th>Medium Pool (%)</th>
<th>Low Pool (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL ng/ml (1)</td>
<td>14.6</td>
<td>7.16</td>
<td>14.9</td>
</tr>
<tr>
<td>T₃ ng/ml (3) *</td>
<td>29.8</td>
<td>-</td>
<td>13.6</td>
</tr>
<tr>
<td>T₄ ng/ml (1)</td>
<td>16.9</td>
<td>17.0</td>
<td>31.3</td>
</tr>
<tr>
<td>T₄ nM/L (3) *</td>
<td>8.4</td>
<td>16.6</td>
<td>17.7</td>
</tr>
<tr>
<td>CORT ng/ml (4) *</td>
<td>14.0</td>
<td>2.9</td>
<td>10</td>
</tr>
<tr>
<td>INS ng/ml (3) *</td>
<td>1.3</td>
<td>-</td>
<td>27.2</td>
</tr>
<tr>
<td>PRL = prolactin, T₃ = triiodothyronine, T₄ = thyroxine, CORT = corticosterone, INS = insulin, GLUC = glucagon, LH = luteinising hormone. The number of assays performed for each hormone is given in brackets. An * indicates that different quality controls were used for all the assays performed.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
10. Molecular techniques

Reagents

0.1% DEPC water
1 ml of diethylpyrocarbonate (DEPC) in 1 L of nanopure water, mixed thoroughly and incubated at room temperature overnight. Autoclaved for 20 minutes at 15 lb/sq inch on liquid cycle to inactivate DEPC.

Luria Bertani (LB) media
To 950 ml nanopure add 10 g tryptone, 5 g yeast extract and 10 g NaCl, adjust volume to 1 L. Sterilise by autoclaving 20 minutes at 15 lb/sq inch on liquid cycle.

LB selective media
Make LB as described. Following autoclaving cool with agitation in a water bath to 60°C and add 100 μg/ml of ampicillin.

LB selective plates
To 950 ml nanopure add 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar, adjust volume to 1 L. Sterilise by autoclaving 20 minutes at 15 lb/sq inch on liquid cycle.

Cool with agitation in a water bath to 60°C and add 100 μg/ml of ampicillin. Pour into sterile plastic petri dishes, allow to set and expose to UV light overnight.

Refrigerate.

10 x TAE
48.4 g tris base, 11.42 ml glacial acetic acid and 20 ml of 0.5 M EDTA pH 8. Adjust volume to 1 L with nanopure water.

10 x TBE
109 g tris base, 55 g boric acid and 40 ml of 0.5 M EDTA pH 8. Adjust volume to 1 L with nanopure water and sterilise by autoclaving.

5% acrylamide gel
14.4 g urea, 3 ml 10 x TBE, 3.8 ml 40% acrylamide (acryl:bis-acryl = 19:1), add 8 ml of dH2O, stir at room temperature until dissolved, adjust volume to 30 ml and degas. While stirring add 240 μl of 10% ammonium persulfate in dH2O and 32 μl of TEMED (Bio Rad). Pour gel immediately.

RNA extraction
RNA was isolated from emu liver, adipose, mediobasal hypothalamus and preoptic area using a method based on that developed by Chomczynski and Sacchi (1987), and a
commercial extraction buffer, RNAzol B (Tel-Test Inc.). Samples were ground in liquid nitrogen using a mortar and pestle resting on dry ice, to prevent RNase activity. The powder was then transferred to an appropriate volume of extraction buffer and processed according to the directions supplied. The methodology was modified to maximise the extraction of RNA from emu adipose, brain and liver tissues. The complete methodology is given below.

**RNAzol B RNA extraction protocol**

Approximately 1 g of the sample tissue was ground to a powder in liquid nitrogen with a mortar and pestle resting on dry ice (for brain tissues the volume of RNAzol was reduced to 5ml). The powder was transferred to a glass grinder containing 10 ml of RNAzol B and homogenised. The homogenate was transferred to a baked 15 ml Corex tube and rested on ice for 5 minutes. A volume of 2 ml of chloroform was added, and the tube vortexed to ensure thorough mixing, the tube was placed on ice for a further 5 minutes. Samples were centrifuged at 12,000 x g and 4°C for 1 hour. The upper aqueous phase was pipetted into a sterile 15 ml glass Corex tube, with care taken not to disturb the interphase, an equal volume of isopropanol was added and mixed by vortexing. Samples were stored at -20°C for more than 45 minutes (for adipose and brain tissues that do not have abundant mRNA’s, samples were stored between 24 and 48 hours to maximise the precipitation of RNA). Tubes were centrifuged at 6,000 x g and 4°C for 45 minutes. The supernatant was removed and the pellet rinsed once with 5 ml of 75% ethanol, followed by centrifugation at 6,000 x g and 4°C for 45 minutes. The supernatant was removed and the pellet air dried for 10-15 minutes. The pellet was dissolved in 1 ml (liver), 0.1 ml (adipose) or 20 μl (mediobasal hypothalamus/preoptic area) of DEPC treated distilled water, with the addition of 1 μl of RNasin RNase inhibitor (Promega) to each sample, and stored at -70°C.

**Ribonuclease Protection Assay (RPA)**

RPA’s for neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) expression in the mediobasal hypothalamus and preoptic area were performed at the Roslin Institute, Scotland, with the assistance of Dr Boswell (Chapters 5 and 6). The technique was then established in our laboratory with the generous gift of emu specific NPY, VIP and β-actin clones. The RPA technique was subsequently used to determine the expression of NPY and VIP in the final experiment (Chapter 7).
Total RNA was extracted from tissues using the methodology given above, and stored under 70% ethanol before being sent to the Roslin Institute (Chapters 5 and 6) or used in our laboratory (Chapter 7). At Roslin, emu specific VIP, NPY and β-actin inserts were ligated into the bluescript plasmid (Stratagene) and transformed into the XL1-blue *Escherichia coli* strain (Stratagene), to be used as probes. The Ambion RPA III™ kit was used to determine NPY and VIP expression using the methodology below:

*Probe and control preparation:*

Cultures of clones containing VIP, NPY and β-actin were grown up overnight in 5 or 10 ml of LB selective media. The vector DNA was extracted using QIAGEN mini-preps (Roslin) or midipreps (UWA) following the manufacturers directions.

Table 2.4: RNA polymerase and restriction enzyme required for each probe to obtain sense and antisense strands.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Antisense strand</th>
<th>Sense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emu VIP</td>
<td>T7; Eco RI</td>
<td>T3; Hind III</td>
</tr>
<tr>
<td>Emu NPY</td>
<td>T7; Eco RI</td>
<td>T3; Hind III</td>
</tr>
<tr>
<td>Emu β-actin</td>
<td>T3; Hind III</td>
<td>T7; Eco RI</td>
</tr>
</tbody>
</table>

*Restriction Digestion:*

The plasmid DNA obtained was digested by adding 20 μl buffer and 10 μl enzyme (Boehringer Mannheim see Table 2.4) to 180 μl plasmid DNA (~40 μg). This was then vortexed and microfuged briefly. The tubes were then incubated at 37°C for 60-90 minutes after which a further 5 μl of enzyme was added and the tubes incubated for a further 2 hours. At this point the samples were either frozen at -20°C or phenol-chloroform extraction performed.

*Phenol-chloroform extraction:*

To each 200 μl digest 100 μl phenol and 100 μl of the chloroform mix (24:1, chloroform:isoamyl alcohol) was added. The tubes were vortexed for 1 minute and microfuged for 2 minutes at maximum rpm. The upper aqueous layer was transferred to a new tube. A volume of 200 μl of dH₂O was added to the original tube and the contents were back extracted by vortexing for 1 minute and microfuging for 2 minutes at maximum rpm. The upper aqueous layer was transferred to the tube containing the first upper layer removed. To this was added 400 μl of the chloroform mix and the tubes
were vortexed for 1 minute and microfuged for 2 minutes at maximum rpm. The upper aqueous layer was transferred to a new tube and the chloroform extraction repeated. To precipitate the digested plasmid DNA 40 \( \mu l \) of 3 M sodium acetate pH 5.2 and 1100 \( \mu l \) of cold 100% ethanol were added. The tubes were vortexed and incubated at -20°C for at least 30 minutes. To pellet the DNA the tubes were microfuged for 20 minutes at 4°C and maximum rpm, and rinsed with 70% ethanol (1 ml added and tubes microfuged for 10 minutes at maximum rpm). The supernatant was removed completely and the pellet air dried for approximately 5 minutes. The sense and antisense plasmid DNA templates were then reconstituted in 32 \( \mu l \) of dH\(_2\)O and the concentration determined spectrophotometrically.

**Preparation of positive control from the sense strand for each probe:**
A total of 1 \( \mu g \) of sense template DNA was denatured for 3 minutes and placed on ice. A nucleotide mix was prepared by combining 2 \( \mu l \) of each supplied nucleotide with 2 \( \mu l \) of dH\(_2\)O. To each of the templates 1 \( \mu l \) of the nucleotide mix, 2 \( \mu l \) of buffer and 2 \( \mu l \) of the appropriate RNA polymerase (Table 2.4) were added and the final volume was adjusted to 20 \( \mu l \) with dH\(_2\)O. The reactions were incubated for 2 hours at 37°C and 2 \( \mu l \) of DNase was added and the tubes incubated for a further 20 minutes at 37°C. To stop the reaction and precipitate the sense strand 2 \( \mu l \) of EDTA (0.2 M), 2.5 \( \mu l \) of 4 M lithium chloride (LiCl) and 75 \( \mu l \) of 100% ethanol were added to the tubes. These were vortexed and incubated at -70°C for greater then 30 minutes or overnight at -20°C. The tubes were microfuged for 20 minutes at 4°C and as much supernatant as possible removed before rinsing with 70% ethanol. The sense strands were reconstituted in 40 \( \mu l \) of deionized formamide and incubated at 70°C for 1 hour. To use as a control 1 \( \mu l \) of sense strand was diluted 1:5000 with dH\(_2\)O immediately prior to performing the reaction.

**Labeling of probes:**
To 0.5 \( \mu l \) of the antisense template DNA, obtained as described above, 0.5 \( \mu l \) nucleotide mix (1 \( \mu l \) each of ATP, CTP, GTP and a 1:200 dilution of UTP + 6 \( \mu l \) of dH\(_2\)O), 1 \( \mu l \) buffer, a trace amount of polymerase (T7 NPY and VIP; T3 β-actin) and 6.75 \( \mu l \) dH\(_2\)O was added. The tubes were microfuged briefly and 1.25 \( \mu l \) [\( \alpha -^{32}\)P]UTP (800 Ci/mmol) was added. The tube contents were mixed by gentle pipetting and incubated for 30 minutes at 37°C. A volume of 1 \( \mu l \) of DNase was added and the tubes incubated for 20 minutes at 37°C. The reaction was stopped and the antisense RNA precipitated by
adding 1 μl of 0.2 M EDTA, 1.25 μl of 4 M LiCl and 37.5 μl of cold 100% ethanol. The tubes were then incubated at -20°C for more than 15 minutes. To pellet the RNA the tubes were microfuged for 15 minutes at 4°C and maximum rpm and the supernatant completely removed. The pellet was rinsed with 1 ml of 70% ethanol and microfuged for 10 minutes, the supernatant was then completely removed and the RNA reconstituted in 100 μl of sterile dH₂O. A volume of 2 μl of this solution was added to scintillant and counted using a beta counter.

Hybridisation:
The hybridisation reagents listed in Table 2.5 were added to a fresh eppendorf tube. The tube contents were mixed thoroughly and incubated at -20°C for more than 15 minutes. The tubes were microfuged at maximum rpm for 15 minutes at 4°C and the supernatant was completely removed. The pellet was resuspended in 10 μl of hybridisation buffer, vortexed and briefly microfuged. Samples were denatured at 90-95°C for 3-4 minutes and then incubated overnight at 42°C.

Table 2.5: Reagents for RPA hybridisation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>+ve control</th>
<th>sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample RNA (μg)</td>
<td>-</td>
<td>-</td>
<td>10-20</td>
</tr>
<tr>
<td>Yeast RNA (μl)</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1:5000 dilution sense</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>strand (μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dH₂O (μl)</td>
<td>28</td>
<td>27</td>
<td>to 30 μl</td>
</tr>
<tr>
<td>NPY Probe</td>
<td>&gt;50,000 cpm</td>
<td>&gt;50,000 cpm of the appropriate probe</td>
<td>&gt;50,000 cpm</td>
</tr>
<tr>
<td>VIP probe</td>
<td>&gt;50,000 cpm</td>
<td></td>
<td>&gt;50,000 cpm</td>
</tr>
<tr>
<td>β-actin probe</td>
<td>&gt;50,000 cpm</td>
<td></td>
<td>&gt;50,000 cpm</td>
</tr>
<tr>
<td>5 M NH₄OAc (μl)</td>
<td>1/10 volume</td>
<td>1/10 volume</td>
<td>1/10 volume</td>
</tr>
<tr>
<td>100% ethanol (μl)</td>
<td>2.5 volumes</td>
<td>2.5 volumes</td>
<td>2.5 volumes</td>
</tr>
</tbody>
</table>

RNase digestion of hybridized probe and sample RNA:
The RNaseA/RNaseT1 was diluted 1:100 with RNase digestion buffer, vortexed and briefly microfuged to ensure even dispersal of components. The hybridisation reactions were briefly microfuged to collect all liquid at the bottom of the tube. A volume of 150 μl of the 1:100 RNaseA/RNaseT1 dilution was added, vortexed and the tubes microfuged briefly. The reactions were incubated for 30 minutes at 37°C. A volume of 225 μl of RNase inactivation/precipitation III solution was added, the tubes vortexed
and microfuged briefly. The reactions were incubated at -20°C for more than 15
minutes. Following incubation the reactions were microfuged at maximum rpm for 15
minutes at 4°C and the supernatant carefully removed. The pellets were resuspended in
8 μl of gel loading buffer II, vortexed vigorously and briefly microfuged. Reactions
were incubated at 90-95°C for 3 minutes, briefly vortexed and microfuged and rested on
ice. The samples were loaded onto a 1 x TBE, 5% acrylamide gel (0.75 mm thick, 12
cm long x 15 cm wide) and run at a constant 250 volts for approximately 1 hour or until
the leading dye band neared the bottom of the gel. Once finished running the gel was
transferred to filter paper, the origins and orientation marked, covered with plastic wrap,
and dried. Once dry the gel was exposed to a phosphoimaging plate overnight at -80°C.
The bands corresponding to β-actin, NPY and VIP were visualised and analysis of their
intensity was performed on a Macintosh (iMac) computer using the public domain NIH
Image program version 1.61 (developed at the U.S. National Institutes of Health, an
available on the Internet at http://rsb.info.nih.gov/nih-image/).
Chapter 3

The emu leptin gene.
1. Introduction

In emus, reproduction is associated with decreased appetite and adiposity. This could be expected to result in parallel decreases in the circulating concentration of leptin. In humans and rodents decreases in the concentration of leptin would stimulate appetite. This clearly does not occur in the emu, since anorexia and diminished appetite are features of incubating and non-incubating animals respectively during the breeding season (Mawson, 1992; Blake, 1996; Blache & Martin, 1999; Blache et al, 2001b). So how is appetite suppressed in emus despite diminishing adipose reserves and what role, if any, does leptin play?

In species that initiate reproduction in response to decreasing day length, seasonal changes in body weight and adiposity, are paralleled by changes in the concentration of leptin (Bocquier et al, 1998; Atcha et al, 2000; Horton et al, 2000; Mercer et al, 2000a; Concannon et al, 2001; Marie et al, 2001). It has been proposed that higher sensitivity to leptin during short days increases the responsiveness to food deprivation and enhance the ability to mobilise adipose reserves in sheep (Bocquier et al, 1998). In the Siberian hamster, leptin administration decreased fat reserves in animals exposed to short but not long photoperiods and had no effect on food intake under either photoperiod (Atcha et al, 2000). The decrease in fat reserves during short photoperiod indicates enhanced sensitivity to the actions of leptin. In this species, food deprivation during short but not long photoperiods decreased the leptin concentration and leptin receptor (Ob-Rb) expression indicating a greater sensitivity to food deprivation (Mercer et al, 2000a).

The involvement of leptin in appetite and adiposity control during the reproductive period could take several forms. From the information available on leptin in other species and our knowledge of the appetite and adiposity changes that occur there are four scenarios that seem likely. First, as adiposity decreases, elevated leptin concentrations are maintained and act to depress appetite. Second, that the leptin concentrations may decrease in parallel with adiposity, and this fails to stimulate appetite as a result of the actions of other factors, possibly related to photoperiod, influencing appetite. Third, that changes in the expression of the leptin receptor at the hypothalamic level alter the responsiveness to leptin. Finally, it is possible that emus do not have the leptin gene or its product. However, this final scenario is unlikely based on evidence for the existence of the leptin gene in chickens. First, southern hybridisations of the mouse leptin gene to chicken DNA indicated
homology between the two species (Zhang et al, 1994). Second, sequences for the chicken leptin gene and chicken leptin receptor have been published (Taouis et al, 1998; Ashwell et al, 1999a; Horev et al, 2000). As leptin is highly conserved in mammalian species it is likely that it is also highly conserved in avian species. As such, the existence of the leptin gene in chickens argues strongly for the existence of a homologous sequence in the emu.

A bioassay for chicken leptin was recently developed by Dr Friedman-Einat based on a human leptin bioassay produced by the Merck Company (Rosenblum et al, 1998). It utilises a firefly luciferase reporter construct and the chicken leptin receptor transfected into the HEK-293 embryonic kidney cell line (Rosenblum et al, 1998). Activation of the leptin receptor by leptin in plasma or serum in turn activates STAT3, a transcription factor, causing the reporter construct to emit light. The amount of light emitted can be quantified and the activity of leptin in the sample deduced. It could be expected that if emu and chicken leptin sequences have been highly conserved that leptin in emu serum will activate the chicken leptin receptor in this assay.

To demonstrate the existence of leptin in emus and in particular its presence in serum, which is the first step in the study of the possible role of leptin in the emu, we tested the following hypotheses;

1. Emus have a leptin gene and its sequence is homologous to the published chicken and mammalian sequences, and
2. Emu serum will activate the chicken leptin receptor in the chicken leptin bioassay.
2. Materials and Methods

Experimental design

The samples used for this experiment were obtained from male and female emus that had been used in observational studies. Several molecular techniques were employed to test the hypotheses outlined in the introduction. First, RNA was extracted from liver and adipose tissue and used or reverse transcription polymerase chain reaction (RT-PCR) with the primers for the chicken leptin gene sequence. Rapid amplification of cDNA ends (RACE) was performed to obtain the complete emu leptin gene sequence. Second, chicken and mammalian leptin probes, were used to screen an emu cosmid library for homologous sequences. Finally, emu serum was added to a cell line containing the chicken leptin receptor to determine if components within the serum could activate the chicken leptin receptor.

Animals and samples

The tissue samples used for this work were obtained from emus of different sexes, at various times of the year. All of the animals were in good health when they were euthanased. The serum samples used in the chicken leptin bioassay were obtained from six male emus used in observational studies by another researcher. These samples were obtained over the course of a year and all of the animals were in good health.

Poly A+ RNA extraction

Poly A+ RNA was obtained using the protocol described by Sambrook et al (1989).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The cloning of the chicken leptin gene was published after the commencement of my Ph.D. (Taouis et al, 1998; Ashwell et al, 1999a). Primers derived for chicken leptin and a chicken leptin probe (supplied by Dr Ashwell) were subsequently used in attempts to clone the emu leptin gene (Ashwell et al, 1999a).

Reverse Transcription (RT)

cDNA was obtained from RNA using M-MuLV reverse transcriptase and 10 x reverse transcription (RT) buffer (New England BioLabs) following the method supplied by the manufacturer. For the initial isolation of a fragment of the leptin gene, a specific reverse primer designed by Nakavisut (1998) (OBR1 - 5'-TACTCCACAG AGTGTTGGGC-3')
was used in the RT reaction. This primer was designed from a region of high homology between all published DNA sequences for the mammalian leptin gene, and also high homology to the published chicken sequence. The reagents listed in Table 3.1 were added to a fresh 200 μl eppendorf tube and incubated at 37°C for 1 hour. The reaction was heated to 90°C for 5 minutes and then placed on ice. These steps were performed using a Hybaid OmniGene thermal cycler. The dNTP’s were obtained from Pharmacia Biotech and the RNasin from Promega.

Table 3.1: Volumes of reagents used in the reverse transcription reactions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x RT buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>RNA</td>
<td>volume to give 2 μg</td>
</tr>
<tr>
<td>dNTP’s (25 mM)</td>
<td>2.0</td>
</tr>
<tr>
<td>OBR1 (100 pmol/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td>RNasin (40 units/μl)</td>
<td>1.0</td>
</tr>
<tr>
<td>M-MuLV (25 units/μl)</td>
<td>4.0</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to a final volume of 50 μl</td>
</tr>
</tbody>
</table>

Polymerase Chain Reaction (PCR)

The PCR was performed using the external sense primer sCLep1 (5’-CACCAGGATCAATGACATTTCAC-3’) (Ashwell et al, 1999a) and the OBR1 primer (Nakavisut, 1998). The reagents listed in Table 3.2 were added to a fresh 200 μl eppendorf tube, adding the Taq DNA polymerase (Promega) last. The 10 x PCR buffer (MgCl₂ free) was supplied with the Taq DNA polymerase.
Table 3.2: Volumes of reagents used in the polymerase chain reactions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT product</td>
<td>10.0</td>
</tr>
<tr>
<td>OBR1 (100 pmol/µl)</td>
<td>0.1</td>
</tr>
<tr>
<td>sCLep1 (10 pmol/µl)</td>
<td>0.5</td>
</tr>
<tr>
<td>10 x PCR buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 units/µl)</td>
<td>0.5</td>
</tr>
<tr>
<td>dH2O</td>
<td>33.9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50.0</strong></td>
</tr>
</tbody>
</table>

Thermal cycling was performed using a Hybaid OmniGene thermal cycler under the conditions given in Table 3.3.

Table 3.3: Thermal cycling conditions used for the polymerase chain reaction.

<table>
<thead>
<tr>
<th>Step</th>
<th>Denature</th>
<th>Anneal</th>
<th>Extend</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C, 1 min</td>
<td>50°C, 1 min</td>
<td>72°C, 2 mins</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>94°C, 30 sec</td>
<td>50°C, 30 sec</td>
<td>72°C, 1 min</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>72°C, 5 mins</td>
<td>1</td>
</tr>
</tbody>
</table>

The products were cooled to 15°C in the thermal cycler before being run on a 1% agarose gel or used directly in nested PCR.

Nested PCR was performed using the primers:

sCLep2 (5'-CGTCGGTATCCGCAAGCAGAGGG-3') and
aCLep2 (5'-CCAGGACGCCATCCAGGCTCTCTGCGC-3') (Ashwell et al, 1999a).

The volumes of the nested PCR reagents used, dNTP's (Pharmacia Biotech), 10 x PCR buffer, MgCl₂, Taq DNA polymerase (Promega), and Triton x 100 (Sigma), are given in Table 3.4.
Table 3.4: Volumes of reagents used in nested PCR.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTP's (25 mM)</td>
<td>0.1</td>
</tr>
<tr>
<td>sCLep2 (10 pmol/µl)</td>
<td>0.1</td>
</tr>
<tr>
<td>aCLep2 (10 pmol/µl)</td>
<td>0.1</td>
</tr>
<tr>
<td>10 x PCR buffer</td>
<td>1.0</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>0.7</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 units/µl)</td>
<td>0.1</td>
</tr>
<tr>
<td>Triton x 100 (diluted 1:100)</td>
<td>0.5</td>
</tr>
<tr>
<td>dH₂O</td>
<td>6.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10.0</strong></td>
</tr>
</tbody>
</table>

Thermal cycling was performed as described in Table 3.3 with an annealing temperature of 55°C in steps 1 and 2.

Purification of PCR products
On the basis of the published leptin gene sequences a 260 base pair (bp) PCR product was expected using these primers. The PCR products were visualised using 1% agarose gels, excised from the gel using a sterile razor blade and extracted with the Qiaex II gel extraction kit (QIAGEN) according to the manufacturers directions.

Cloning
Reagents

_Luria-Bertani (LB) media and plates (with or without ampicillin)_

The media and plates were made according to the directions of Sambrook _et al_ (1989).

Competent cells
Competent cells were prepared according to the methodology of Sambrook _et al_ (1989). Briefly, _Escherichia coli_ (E. coli) of the XL1-blue strain were streaked onto LB media plates (no ampicillin) and incubated overnight at 37°C. A single colony was selected from the plate and used to inoculate 5 ml of LB medium. The culture was grown overnight at 37°C with gentle agitation. This culture was diluted 1:100 with 500 ml of LB media pre-
warmed to 37°C and was incubated at 37°C with vigorous shaking to an OD$_{650}$ of 0.8. The cells were chilled quickly by swirling gently in an ice water bath for approximately 5 minutes. The cells were centrifuged at 8000 rpm for 8 minutes at 4°C. The supernatant was poured off and the pellet resuspended in 125 ml of ice-cold 0.1M MgCl$_2$ and immediately centrifuged as described above. After pouring off the supernatant the cells were resuspended in 125 ml of ice-cold 0.1M CaCl$_2$ and incubated on ice for 20 minutes, before being centrifuged as described above. After pouring off the supernatant the cells were resuspended gently in 25 ml of ice-cold 0.1M CaCl$_2$ containing 14% v/v glycerol, and the suspension was incubated on ice for less than 30 minutes. The competent cells were aliquoted in 0.2 ml volumes into pre-chilled eppendorf tubes and snap frozen in liquid nitrogen. The cells were then stored at -80°C.

Ligation of the purified PCR product into the pGEM-T easy vector.

The purified nested PCR product was run on a 1% agarose gel and a visual assessment with Kodak ID20 software was used to estimate the concentration. Ligation of the purified product into a vector was performed using the pGEM-T easy kit (Promega) (Table 3.5). An insert to vector ratio of 3:1 was used where possible.

Table 3.5: Volumes of reagents used in the ligation reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x ligase buffer</td>
<td>1</td>
</tr>
<tr>
<td>Ligase (3 units/µl)</td>
<td>1</td>
</tr>
<tr>
<td>pGEM-T (50 ng/µl)</td>
<td>1</td>
</tr>
<tr>
<td>insert</td>
<td>~150 ng</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>to a final volume of 10 µl</td>
</tr>
</tbody>
</table>

The ligation was prepared on ice and allowed to proceed either overnight at 4°C, or at 15°C for a minimum of 2 hours.

The ligated product was transformed into the *E. coli* strain XL-1 blue using the heat shock method (Sambrook *et al*, 1989). Briefly, competent cells were thawed on ice and 2 µl of the ligation reaction was added. The reaction was incubated on ice for 30 minutes. The reaction tube was transferred to a waterbath at 42°C for 90 seconds and returned to ice for
1-2 minutes. A volume of 800 μl of LB media was added to the reaction tube and incubated at 37°C for 90 minutes in a shaking incubator. The reaction was microfuged at 5000 rpm for 5 minutes and 800 μl of LB media was removed with a pipette. The pellet was resuspended in the remaining LB media and diluted to 1:10 and 9:10, and spread onto LB plates containing ampicillin. The dilutions used allowed individual colonies to be selected after the incubation period. The presence of ampicillin in the media allowed *E. coli* colonies containing the pGEM-T vector to be identified as it confers ampicillin resistance. The inoculated plates were incubated at 37°C overnight.

Colony PCR.

Colony PCR was used to determine colonies that harboured inserts of the desired size. Briefly, individual colonies that had grown on the selective plates were touched with a sterile sealed glass pasteur pipette (Chase Instruments), which was then touched to a fresh selective plate with an identifying grid drawn on the petri dish. After this the tip of the glass rod was placed in an eppendorf tube with 10 μl of sterile distilled water and agitated gently to produce a bacterial suspension. The tube was numbered to correspond with the colony’s grid location on the new plate. The SP6 and T7 primers were used to identify colonies with inserts of the correct size, as they anneal either side of the multiple cloning region of the pGEM-T vector. The volumes of PCR reagents, dNTP’s (Pharmacia Biotech), 10 x PCR buffer, MgCl2 and Taq DNA polymerase (Promega), used in the colony PCR are given in Table 3.6.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial suspension</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTP’s (25 mM)</td>
<td>0.1</td>
</tr>
<tr>
<td>SP6 (10 pmol/μl)</td>
<td>0.1</td>
</tr>
<tr>
<td>T7 (10 pmol/μl)</td>
<td>0.1</td>
</tr>
<tr>
<td>10 x PCR buffer</td>
<td>1.0</td>
</tr>
<tr>
<td>MgCl2 (25 mM)</td>
<td>0.7</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 units/μl)</td>
<td>0.1</td>
</tr>
<tr>
<td>dH20</td>
<td>6.9</td>
</tr>
<tr>
<td>Total</td>
<td>10.0</td>
</tr>
</tbody>
</table>
The thermal cycling conditions for the colony PCR are as given in Table 3.3, with the exception that the annealing temperature used at step 2 was 55°C. The colony PCR products were run on a 1% agarose gel and those ascertained to have inserts of the desired size were grown up in 5 ml of LB + ampicillin media (mini culture) for about 8 hours. This was diluted 1:100 into 25 ml of LB + ampicillin media (maxi culture) and grown up overnight. The remainder of the mini culture was used to make glycerol stocks by adding 500 μl of the culture and 500 μl of 80% glycerol (Sigma) to a sterile eppendorf tube that was then stored at -80°C. The plasmid midifilter kit (QIAGEN) was used to obtain plasmid DNA according to the manufacturer’s directions from the maxi culture. After assessing the resulting plasmid DNA concentration and quality spectrophotometrically, it was sequenced at the Immunology Department of Royal Perth Hospital. The GeneJockey II program was utilised to find regions of the sequence corresponding to SP6, T7 and the primers used in the nested PCR. Once the regions amplified by these were found they were compared to other known leptin gene sequences with the GeneJockey II program and also utilising the BLAST service offered by the National Institute of Health, USA, accessed via the internet (http://www.ncbi.nlm.nih.gov/).

Cosmid library screening
An emu cosmid library (Stratagene) (Hammond, 2000) was screened with a 260 bp mammalian leptin fragment and Ashwell’s chicken leptin fragment labelled with [α³²P] dCTP (NEN).

Restriction digestion of plasmid DNA
The plasmid DNA of both leptin fragments were digested with restriction enzymes to remove the vector DNA as this shares homology with cosmid DNA used in the emu cDNA library. The volumes of EcoRI, 1 x NE buffer for EcoRI (New England Biolabs), BSA, NcoI, SalI and 10 x buffer D (Promega) used in the restriction digest are given in Table 3.7. Ashwell’s chicken leptin fragment was digested with EcoRI and the mammalian fragment with NcoI and SalI by adding the reagents as indicated in Table 3.7, mixing them well by pipetting and incubating overnight at 37°C.
Table 3.7: Volumes of reagents used for restriction digests of plasmids containing chicken or mammalian leptin fragments.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Chicken leptin (µl)</th>
<th>Mammalian leptin (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (10 µg)</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>BSA (acetylated 10 mg/ml)</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>EcoRI</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>NcoI</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>SalI</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>1 x NE buffer for EcoRI</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>10 x buffer D</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>26.5</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

The restriction digest products were precipitated by adding 5 µl of 3 M sodium acetate (pH 5.2) and 165 µl of absolute ethanol. The reagents were mixed thoroughly and stored at -20°C for at least one hour. The reactions were centrifuged at 12,000 g and the supernatant removed completely. The pellets obtained were resuspended in 15 µl of sterile distilled water. The entire volume was then loaded onto a 1% agarose, 1 x TBE gel and run for approximately one hour at 80 volts. The 100 bp marker (Promega) was also run with the restriction products and a band of approximately 300 bp was excised and purified with the Qiaex II gel extraction kit (QIAGEN) as described previously.

**Labelling probes**

The chicken and mammalian leptin probes were labelled using the New MEGAPRIME kit, protocol 1606 (Amersham). Briefly, the insert DNA concentration was diluted to 5 ng/µl with distilled water. Using this dilution 25 ng of the DNA insert was added to a sterile eppendorf tube with 5 µl of the supplied primers, and denatured by heating to 95-100°C for 5 minutes in a water bath. The tube was microfuged briefly. At room temperature, 10 µl of labelling buffer, 2 µl of enzyme and 23 µl of distilled water were added to the tube. The tube was microfuged briefly and 5 µl of [α-32P] dCTP (NEN), specific activity 3000 Ci/mmol, was added and mixed by gentle pipetting, followed by brief microcentrifugation. The tube was incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 5 µl of 0.2M EDTA and denatured by heating to 95-100°C for 5 minutes. The labelled insert was chilled on ice before being put through a NICK column (Pharmacia Biotech) to
remove unincorporated nucleotide, according to the manufacturers directions.

Approximately 100 µl fractions were collected and those with the highest levels of radioactivity were identified with a geiger counter (Morgan series 900 mini monitor) and pooled.

Southern Hybridisation
Reagents

*Salmon sperm (SS) DNA*

The SS DNA was prepared according to the directions of Sambrook *et al* (1989).

*10 x PIPES (piperazine-N,N’-bis[2-ethane sulfonic acid])*  
To 700 ml of sterile distilled water 30.24 g of PIPES was added, and the pH was adjusted to 6.5 with 0.1 N HCl if required. To this was added 233.76 g of NaCl. Sterile distilled water was added to give a final volume of 1 L and a final concentration of 4 M NaCl and 0.1 M PIPES (pH 6.5).

*20 x SSC*

To 800 ml of distilled water 175.3 g of NaCl and 88.2 g of sodium citrate were added. The pH was adjusted to 7 with 10 N NaOH and distilled water added to give a final volume of 1 L with. The solution was sterilised by autoclaving on a liquid cycle (15 lb/sq inch) for 20 minutes.

*10% SDS*

To 900 ml of distilled water 100 g of electrophoresis grade SDS was added. The solution was heated to 68°C to assist dissolution. The pH was adjusted to 7.2 with concentrated HCl and distilled water added to give a final volume of 1 L.

*1M Tris-Cl (pH 7.6)*

To 800 ml of distilled water 121.1 g of Tris base was added, the pH was adjusted to 7.6 with concentrated HCl and distilled water added to give a final volume of 1 L. The solution was sterilised by autoclaving on a liquid cycle (15 lb/sq inch) for 20 minutes.
$1M$ Tris-Cl (pH 7.6) + $1.5M$ NaCl
Made as for $1M$ Tris-Cl (pH 7.6) with the addition of NaCl to a final concentration of $1.5M$.

$0.5M$ NaOH
To 800 ml of distilled water 20 g of NaOH was added. This was allowed to dissolve and the final volume was adjusted to 1 L with sterile distilled water.

Cosmid prehybridisation solution ($2x$ PIPES, 50% deionized formamide, 0.5% SDS (w/v))
A volume of 200 ml of 10 x PIPES, was added with 500 ml of deionized formamide and 50 ml of 10% SDS to a sterile 1 L Schott bottle. The final volume was adjusted the to 1 L with sterile distilled water.

$2x$ SSC + 0.1% SDS
This solution was prepared by diluting 20 x SSC 1 in 10, and 10% SDS 1 in 100 with sterile distilled water to give a final volume of 1 L in a sterile Schott bottle.

$Ix$ SSC + 0.1% SDS
This solution was prepared by diluting 20 x SSC 1 in 20, and 10% SDS 1 in 100 with sterile distilled water to give a final volume of 1 L in a sterile Schott bottle.

Southern hybridisation to the emu cosmid library
The emu cosmid library was spread on to LB + ampicillin media plates in 200 μl volumes at $1 \times 10^6$ and $1 \times 10^7$ dilutions, and grown up overnight at 37°C. Replica membranes were made by placing a Hybond-N+ (Amersham) membrane on each plate and allowing them to wet through. The membranes were then removed and placed colony side up onto fresh LB + ampicillin media plates and grown for a further four hours at 37°C.

Positive control membranes were prepared by denaturing plasmid DNA containing the mammalian and chicken leptin gene sequences at 95°C for 5 minutes before dotting onto Hybond-N+ membrane (Amersham). The membrane was allowed to air dry and then baked at 80°C for one hour to fix the DNA.
The replica membranes were prepared for hybridisation by placing them colony side up for 30 seconds onto a series of number 3 Whatman papers (Whatman International Ltd) prewetted with the following solutions:

1. 0.5 M NaOH,
2. 1 M Tris-HCl (pH 7.6), and
3. 1 M Tris-HCl (pH 7.6) + 1.5 M NaCl.

The membranes were placed in a solution of 1 M Tris-HCl (pH 7.6) + 1.5 M NaCl and any colony debris was gently removed by rubbing a gloved hand over the surface of the membrane. The membrane was rinsed in fresh 1 M Tris-HCl (pH 7.6) + 1.5 M NaCl and allowed to air dry on paper towel before being baked at 80°C for one hour.

The replica and positive control membranes were placed on fine nylon gauze prewetted with the cosmid prehybridisation solution, this was gently rolled up and placed in Hybaid HB-OV-BM hybridisation bottles. A volume of 20 ml of the cosmid prehybridisation solution pre-warmed to 50°C was added to the bottle with 200 µl of salmon sperm DNA that had been boiled for 10 minutes immediately prior to its addition. The hybridisation bottle was sealed and the membranes were prehybridised at 46°C for a minimum of two hours in a Hybaid mini oven. The probes were prepared as described previously and at the completion of prehybridisation boiled at 95-100°C for 5 minutes and added to the prehybridisation solution. Hybridisation was carried out overnight at 46°C in a Hybaid mini oven. At the completion of hybridisation the solution was poured off and 20 ml of 2 x SSC + 0.1% SDS, pre-warmed to 60°C, was added to the bottles. The membranes were washed for 5 minutes in a Hybaid mini oven, and then the solution was poured off and the wash repeated twice. A final wash was performed for 30 minutes with 20 ml of 1 x SSC + 0.1% SDS pre-warmed to 60°C. A geiger counter (Morgan series 900 mini monitor) was used to measure the background radiation on the membrane and the wash steps repeated if this was deemed too high. The membranes were blotted on paper towel and wrapped in cling film before being exposed to x-ray film (Kodak scientific imaging film) overnight at -80°C. The film was developed using Kodak chemicals according to the manufacturers directions.

**Rapid amplification of cDNA ends (RACE)**

Following the amplification of an appropriately sized band from emu liver RNA with leptin gene primers that were specific for the chicken (Ashwell *et al*, 1999a) Clontech's
Marathon RACE cDNA amplification kit was employed to obtain the remainder of the 3' and 5' ends of the sequence from poly A+ emu liver RNA.

The protocols outlined for the Marathon kit were followed, with the exception of the thermal cycling conditions, which were optimised for the Hybaid OmniGene thermal cycler and are given in Table 3.8.

**Table 3.8:** The thermal cycling conditions for the Marathon RACE reactions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Denature</th>
<th>Anneal</th>
<th>Extend</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C, 30 sec</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>94°C, 30 sec</td>
<td>65°C, 1 min</td>
<td>68°C, 3 mins</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>94°C, 30 sec</td>
<td>60°C, 1 min</td>
<td>68°C, 3 mins</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>94°C, 30 sec</td>
<td>57°C, 1 min</td>
<td>68°C, 3 mins</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>72°C, 5 mins</td>
<td>1</td>
</tr>
</tbody>
</table>

The four largest bands resulting from the RACE reaction were excised from the gel and purified using the Qiaex II kit (QIAGEN). PCR was performed on the products using the nested primers reported by Ashwell *et al* (1999a) and the thermal cycling conditions described in Table 3.3, with the exception that annealing at steps 1 and 2 were performed at 55°C. Any fragments that were approximately 260 bp in length were ligated into pGEM-T and transformed into the *E. coli* strain XL1-blue, using the methodology described previously. The resulting colonies were screened using the SP6 and T7 primers to determine which clones contained inserts of the same size as the RACE product. These were screened further via the nested PCR reaction as above. Those colonies that contained plasmids with inserts of the correct size were grown up to make glycerol stocks as described previously and for plasmid DNA preparations using the midifilter kit (QIAGEN) according to the manufacturer's directions. The quality and concentration of the plasmid DNA were determined spectrophotometrically. Plasmids were sequenced at the Immunology Department of Royal Perth Hospital.

**Chicken leptin bioassay**

This work was performed with Dr Friedman-Einat at the Institute of Agriculture, Volcani Center, Israel. The bioassay utilises a firefly luciferase reporter construct and the chicken leptin receptor transfected into the HEK-293 embryonic kidney cell line using methods...
reported by Rosenblum et al (1998). The colonies transfected with the leptin receptor were screened by Dr Friedman-Einat to detect the clone with the highest level of leptin-induced luciferase activity. This clone (clepr210) was used to determine the biological activity of leptin in serum samples from emus and chickens. The methodology is given briefly below:

Reagents

*DMEM medium*

10% fetal calf serum, 50 μg/ml streptomycin and 50 U/ml penicillin

*Growth medium*

DMEM + 2 μg/ml puromycin

Culture plate preparation

Growth medium was gently aspirated from a culture stock of clepr210, leaving a layer of cells. An aliquot of 300 μl of trypsin was added and allowed to spread across the plate. Clumps of cells that formed were detached by gentle tapping. The cells were removed from the bottom of the plate by adding 9 ml of DMEM medium and gently pipetting the solution. An aliquot of 1 ml of the resulting solution was added to a new petri dish, and an additional 8 ml of growth medium was added, mixed gently and incubated at 37°C in a 95% air, 5% carbon dioxide atmosphere. A volume of 18 ml of DMEM medium was added to the remaining 8 ml of cell culture. While gently mixing this solution, aliquots of 500 μl (24 well Sarstedt) or 900 μl (6 well culture plate) were distributed into each well of the culture plates (~50,000 cells/well). The plates were then incubated at 37°C in a 95% air, 5% carbon dioxide atmosphere overnight.

Assessment of leptin activity in serum or plasma

The medium was aspirated gently and replaced with OptiMEM serum free medium (GIBCO) pre-heated to 37°C (300 μl for 24 well or 600 μl for 6 well culture plates). The serum samples (20-30 μl emu serum for 24 well or 100 μl for 6 well culture plates) were tested in duplicate. A blank reaction was prepared in duplicate by adding only OptiMEM serum free medium (GIBCO). The culture plates were incubated for 8-16 hours at 37°C in a 95% air, 5% carbon dioxide atmosphere. The medium was poured off and allowed to drain. Once dry, the cells were lysed by the addition of 100 μl of lysis buffer (Promega) and vortexing (24 well culture plates). For the six well culture plates the same volume of
lysis buffer was used and after vortexing the bottom of the wells were scraped to ensure that all cells were lysed. The lysed product was transferred to an eppendorf tube and stored at -80°C. An aliquot of 40 μl was removed for luminometric determination. Protein determination was performed in triplicate with 1μl of the lysed product.

Luminometric determination
A volume of 40 μl of luciferase was added to the 40 μl aliquot of lysed cells immediately prior to reading each sample. Tubes were tapped gently and quickly to mix and remove air bubbles. The samples were then aliquoted into a sample tube and the light emitted was quantified with a TD20e luminometer (Turner Design Inc.).

Protein determination
The protein concentration of each well was determined by Bradford assays using a 150-fold dilution of each cell lysate. The assays were performed in duplicate. A standard curve was set up using 0, 1, 2, 3, 4 and 5 μl of BSA (1 mg/ml) in duplicate.

Leptin receptor (LepR) induction
The luciferase activity and protein content measurements were used to calculate the luciferase activity per μg of protein in each sample. These values indicate how much the leptin receptors in the cell line were activated by leptin in the samples. To make comparisons between individual bioassays the LepR induction of the samples were expressed as proportions of their blank (Optimem serum free medium only) reaction values. The calculations are as follows:
Sample LepR induction = (luciferase activity / μg of protein) / blank LepR induction
Blank LepR induction = luciferase activity / μg of protein

Data and statistical analysis
The significance of differences over the course of a year, were determined using the Friedman two-way analysis of variance by ranks described by Siegel (1956). Non-parametric statistical analysis was judged the most appropriate, as related samples were used and the sample size was small (n = 6). The results are indicated as the mean ± s.e.m.
3. Results

RT-PCR

A leptin-like product was amplified from emu RNA. The homology of the emu derived product was highly homologous with the ovine leptin gene sequence (98%).

Cosmid library screening

The chicken leptin fragment provided by Dr Ashwell failed to hybridise to the emu cDNA cosmid library. A mammalian leptin gene fragment was used as a positive control in the southern hybridisations. This showed a high degree of homology with Ashwell’s chicken fragment, even after high stringency washing.

RACE

Using either total or poly A+ RNA from emu liver the sCLEp1 and aCLEp1 primers failed to amplify any DNA fragments that resembled the leptin gene (Ashwell et al, 1999a). The highly conserved mammalian OBR1 primer and the chicken specific sCLEp1 primer also failed to amplify the emu leptin gene sequence (Nakavusit, 1998; Ashwell et al, 1999a).

Chicken leptin bioassay

The emu serum samples used in the chicken leptin bioassay produced quantifiable amounts of light, which indicates that components in the samples activated the chicken leptin receptor (Table 3.9 and 3.10). Lower values for the leptin receptor induction were obtained with emu samples than are obtained for the chicken. Samples collected from emus during the course of a year indicated that the biological activity of leptin did not differ in the emu over this period (Table 3.10).

<table>
<thead>
<tr>
<th>Table 3.9: Leptin receptor induction of the chicken leptin bioassay by emu and chicken serum samples.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ratio of sample to blank</strong></td>
</tr>
<tr>
<td>Emu</td>
</tr>
<tr>
<td>2.72 ± 0.64</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m.
Table 3.10: Leptin receptor induction of the chicken leptin receptor bioassay by emu serum samples collected over the course of a year.

<table>
<thead>
<tr>
<th></th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of sample to blank</td>
<td>2.59 ± 0.78</td>
<td>1.93 ± 0.70</td>
<td>2.31 ± 0.94</td>
<td>2.14 ± 0.79</td>
<td>2.86 ± 1.09</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m.
4. Discussion

The first hypothesis, that emus have a leptin gene sequence, is supported by the results. However, the results do not support a high degree of homology between the published chicken and mammalian sequences. The second hypothesis is supported by the activation of the chicken leptin receptor by components in emu serum. In addition activation of the chicken receptor by emu serum provides the evidence that the leptin gene is present in the emu. The ability of emu serum to activate the chicken leptin receptor also suggests that emu and chicken leptins share a high degree of homology. A leptin-like product was amplified using RT-PCR from both chicken and emu RNA that shared high homology with the ovine leptin gene sequence (98%). However, ovine RNA and DNA are regularly used in our laboratory, and other approaches such as RACE and screening of the emu cosmid library failed to identify emu leptin. As such, the product amplified by RT-PCR was deemed to be the result of contamination from aerosols within the laboratory. The failure of RT-PCR, RACE and screening of the emu cosmid library to identify the emu leptin gene would indicate that the emu sequence does not share a high degree of homology with Ashwell et al’s (1999a) chicken or the published mammalian leptin gene sequences. Controls used while screening the emu cosmid library revealed a high degree of homology between the mammalian sequence and Ashwell et al’s (1999a) chicken leptin gene sequence. This is unexpected, as generally genes are more highly conserved between avian species than between avian and mammalian species. These results could be taken to indicate that the published chicken leptin gene sequence is not correct. Other researchers have also concluded that the published sequence for the chicken leptin gene is not correct, and is instead a contamination artefact (Friedman-Einat et al, 1999; Varma, 2000; Dunn et al, 2001). The problem of contamination artefacts being produced using the PCR technique is well documented (Sarkar & Sommer, 1990; Erlich et al, 1991; Neumaier et al, 1998; Burkardt, 2000; Urban et al, 2000).

There are several approaches that can be taken to determine if the published chicken leptin gene sequence is correct. First, sequencing of the chicken and emu leptin gene sequences using alternative methods to those employed by Ashwell et al (1999a) and Taouis et al (1998). A group led by Dr Friedman-Einat at the Volcani Centre is currently attempting this using their chicken leptin receptor sequence to capture the protein ligand and obtain its N-terminal amino acid sequence (Horev et al, 2000). Once the N-terminal amino acid
sequence is known degenerate oligo primers can be designed. PCRs can then be performed using these primers to obtain the complete chicken leptin gene sequence. Second, testing of the protein product of the published chicken leptin gene sequence in the chicken leptin bioassay. The failure of this product to activate the receptor could indicate that the published chicken leptin gene sequence is incorrect. Third, the sequence of the emu leptin receptor gene could be determined. If a high degree of homology between the leptin receptor genes from emu and chicken is found, it will argue strongly for sequence conservation of the leptin gene.

The activation of the chicken leptin receptor by components within emu serum was much lower than observed for the chicken. This could be due to lower specificity of the assay for emu leptin or to lower biological activity of emu leptin. The bioassay also indicated that the concentration of leptin did not differ over the course of a year, again this may be the result of lower specificity of the assay for emu leptin. If in the emu, leptin concentrations increase when adiposity increases and suppress appetite, it would be impossible for the emu to deposit large amounts of fat over the summer months as part of its annual cycle. So, my results suggest that leptin is not involved in appetite regulation in emus in the same manner as it is in other species. The role of leptin in the control of appetite and adiposity in the emu will only be determined once effective methods for measuring leptin concentrations in emu serum are developed. In the following chapters I will present experiments aimed at improving the understanding of how appetite and adiposity are controlled in the emu.
Chapter 4

The influence of dietary fat on food intake and adiposity
1. Introduction

Seasonal fluctuations in feed intake and body weight are observed in emus and correlate directly with their degree of adiposity (Mincham et al., 1998; Blache & Martin, 1999; Blache et al., 2001b). Adiposity and appetite are decreased during the breeding season and increased during the summer months. Decrease in appetite and adiposity during the breeding season could be prevented by dietary interventions that disrupt energy balance. For example, in chickens, feeding diets that are high in fat increases adiposity in proportion to the diet's fat content (Edozien & Switzer, 1978; Deaton et al., 1981; Boozer et al., 1995; Vilà & Esteve-Garcia, 1996). In mammals, high fat diets also induce hyperphagia, although this effect is variable between species and strains and may be transient (Oscai et al., 1984; West et al., 1992; Hill et al., 1992; French et al., 1995; Smith et al., 1998). The variability of hyperphagia in response to high fat diets may be due to the action of high fat diets to decrease the rate of stomach emptying (Mateos et al., 1982). A decreased rate of stomach emptying would make the animal feel sated for longer.

Diets high in fat disrupt energy balance by decreasing the metabolic rate and lowering energy expenditure (Black et al., 1949; Storlien et al., 1986). The mechanism enabling this could involve metabolic changes that can be assessed by measuring hormone concentrations (Ramirez et al., 1990; Ahrén & Scheurink, 1998). When energy intake exceeds the organism's requirements insulin rises, glucagon falls, and metabolism shifts from catabolism of stored energy reserves to anabolism and storage of ingested energy (Norman & Litwack, 1987). Depression of the thyroid hormones reduces the metabolic rate and energy expenditure via depressed body temperature (Norman & Litwack, 1987).

This experiment was performed to determine if feeding diets that are high in fat to emus during the summer months, when appetite is high, can further increase appetite and adiposity. The following hypotheses were formulated:

1. Feeding emus a high fat diet for an extended period will increase adipose tissue deposition and induce hyperphagia, and
2. Feeding emus a high fat diet will alter the concentration of metabolic hormones to favour adipose deposition. This should result in higher concentrations of insulin and lower concentrations of glucagon, triiodothyronine and thyroxine.
2. Materials and Methods

Experimental design
To test the hypotheses male emus were allocated into two groups to be fed either a high (23.6%) or a low (4.5%) fat diet. Changes in appetite and adiposity were assessed by measuring feed intake over the course of the experiment and performing carcass composition analysis on the birds at the completion of the experiment. The concentration of the insulin, glucagon, triiodothyronine and thyroxine were measured over the course of the experiment by radioimmunoassay in order to determine if metabolism had changed to favour adipose deposition.

Animals
Ten male emus (nine 3 year olds and one 4 year old) were penned individually, as described in Chapter 2. They were allocated into two groups of similar average weights (means ± s.e.m. - low fat diet group 41.5 ± 1.5 kg, high fat diet group 42.8 ± 3.1 kg).

Treatments
The birds were offered diets differing in fat, but not protein content (Table 4.2). All birds received a 4.5% fat diet (low fat) for a period of two weeks (Weeks -2 to 0). At the end of the two weeks one group was given a 23.6% fat diet (high fat) for a period of 54 days. Due to difficulties with emus accepting changes in their feed, the switch to the high fat diet was achieved by adding the high fat diet in increasing proportions to the low fat diet, over the first two weeks (Weeks 1 and 2) of the treatment period.

Feeds were made up weekly or as required, with the addition of between 3 and 5% water to the mix to aid in mixing and pelleting (Table 4.1). Flaked barley was reduced to a powder using a grinding mill (Horwood Bagshaw Ltd) and the feed was mechanically mixed in a Barrow Linton (Perth) mixer. Pellets were made with a 5 mm diameter dye using a Lister pellet press. The size of the pellets approximates the size of the commercial breeder pellet that the birds had previously been fed.
Chapter 4 – The influence of dietary fat on food intake and adiposity

Table 4.1: Composition of the low and high fat diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Diet (per 100 kg)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low fat</td>
<td>high fat</td>
<td></td>
</tr>
<tr>
<td>Soya bean meal</td>
<td>45.3 kg</td>
<td>25.6 kg</td>
<td></td>
</tr>
<tr>
<td>Full-fat soya bean meal (Bayer XT-Soya)</td>
<td>3.4 kg</td>
<td>9.1 kg</td>
<td></td>
</tr>
<tr>
<td>Full-fat canola seed (Davison Industries)</td>
<td>5.2 kg</td>
<td>52.7 kg</td>
<td></td>
</tr>
<tr>
<td>Barley flaked</td>
<td>43.1 kg</td>
<td>10.2 kg</td>
<td></td>
</tr>
<tr>
<td>Tricalcium phosphate</td>
<td>1.5 kg</td>
<td>1.5 kg</td>
<td></td>
</tr>
<tr>
<td>Limestone</td>
<td>1.2 kg</td>
<td>0.7 kg</td>
<td></td>
</tr>
<tr>
<td>Ethoxyquin</td>
<td>4.96 g</td>
<td>25 g</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (50%)</td>
<td>4 g</td>
<td>20 g</td>
<td></td>
</tr>
<tr>
<td>Vitamin A/D (100%)</td>
<td>2 g</td>
<td>2 g</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>141.1 g</td>
<td>220 g</td>
<td></td>
</tr>
<tr>
<td>Brilliant blue food dye (Educational colours Pty Ltd)</td>
<td>200 ml</td>
<td>150 ml</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Macronutrient and energy content of the low and high fat diets.

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>low fat</th>
<th>high fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (%)</td>
<td>4.5</td>
<td>23.6</td>
</tr>
<tr>
<td>Fibre (%)</td>
<td>6.0</td>
<td>5.6</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>27.5</td>
<td>27.5</td>
</tr>
<tr>
<td>Total carbohydrate (%)</td>
<td>50.9</td>
<td>30.9</td>
</tr>
<tr>
<td>ME (MJ/kg)</td>
<td>11.4</td>
<td>15.5</td>
</tr>
<tr>
<td>E:P ratio</td>
<td>0.42</td>
<td>0.56</td>
</tr>
</tbody>
</table>

The carbohydrate content of the diet was not controlled and was higher in the low fat diet. The fibre and total carbohydrate contents were estimated from data available for components of the diet (McDonald et al, 1981).

Food intake
Food intake was recorded throughout the experiment, as described in Chapter 2, with food and water offered *ad libitum*.

Live weight and blood sampling
On Days -14, 0, 14, 28, 37 and 54 all birds were weighed and a 10 ml blood sample collected, as described in Chapter 2.
Tissue collection and carcass composition
At the completion of the experiment (Day 56), birds were weighed and bled, prior to being euthanased. Samples of liver and muscle were collected for analysis of fat content. The empty carcass, subcutaneous and visceral fat depots, and the liver were weighed to determine differences in adiposity. The methodology for these procedures is given in Chapter 2.

Hormones and metabolites
Radioimmunoassays were performed for insulin, triiodothyronine, thyroxine and glucagon for all samples as described in Chapter 2.

Data and statistical analysis
The carcass composition data for each tissue is expressed as a percentage of the live weight of the animal. The significance of any differences between treatment groups, were determined using Mann-Whitney U tests as described by Siegel (1956) using the Statview™ 512+ program (1986). This statistical analysis was judged to be the most appropriate method to analyse the data, as two independent groups were sampled from and the sample size was small (n ≤ 5). The significance of any differences within diets over time, was determined using the Friedman two-way analysis of variance by ranks as described by Siegel (1956). This statistical analysis was judged to be the most appropriate as the samples were related and there were more than two time-points involved. The results are indicated as the mean ± s.e.m.
3. Results

Feed Intake

There was a tendency for total feed and protein intakes to be lower on the high fat diet (p = 0.075). The total amount of fat consumed was higher, and the total amount of fibre consumed was lower in birds fed the high fat diet than in birds fed the low fat diet (p = 0.004, p = 0.048 respectively). Total energy consumption did not differ between the two groups (Table 4.3), despite the higher energy content of the high fat diet (Table 4.2).

Table 4.3: Total feed, protein, fibre, fat and energy intakes over 68 days of male emus fed low and high fat diets.

<table>
<thead>
<tr>
<th></th>
<th>low fat diet</th>
<th>high fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total feed intake (kg)</td>
<td>55.3 ± 8.0</td>
<td>39.9 ± 3.0</td>
</tr>
<tr>
<td>Total protein intake (kg)</td>
<td>15.2 ± 2.2</td>
<td>11.0 ± 0.8</td>
</tr>
<tr>
<td>Total fibre intake (kg)</td>
<td>3.34 ± 0.48*</td>
<td>2.30 ± 0.17*</td>
</tr>
<tr>
<td>Total fat intake (kg)</td>
<td>2.51 ± 0.36*</td>
<td>6.50 ± 0.46*</td>
</tr>
<tr>
<td>Total energy consumption (MJ)</td>
<td>631 ± 92</td>
<td>449 ± 40</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference (p < 0.05) between treatment groups.
For the majority of the experiment mean daily feed intake did not differ between the groups. There were two exceptions with mean daily feed intake for Week 1 and Week 2 being lower for the high fat diet group (p = 0.008 and p = 0.028 respectively) (Fig. 4.1). This period is also the transition period between the low fat and high fat diets and may represent an initial rejection of the high fat diet. Within both the low and high fat diet groups feed intake differed over time (p < 0.05 and p < 0.001 respectively).

Figure 4.1: Average daily feed intake for each week of the experiment for male emus receiving either the low (open circle) or high fat diet (solid circle) for 8 weeks. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m. An * indicates significant difference between treatment groups (p < 0.05).
**Live weight**

There was no difference in live weight between the high and low fat diet groups at any time point and no effect of time on live weight within either group (Fig. 4.2).

**Figure 4.2:** Mean live weight of male emus offered low (open circle) and high fat (solid circle) diets over a 54 day treatment period. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m.
Carcass composition

There was no difference in the fat content of either muscle or liver between male emus receiving either diet (Table 4.4).

Table 4.4: The fat content of muscle and liver tissue from male emus fed either low or high fat diets for 54 days.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>low fat</th>
<th>high fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle (g/kg of wet tissue)</td>
<td>4.93 ± 0.41</td>
<td>3.52 ± 0.90</td>
</tr>
<tr>
<td>Liver (g/kg of wet tissue)</td>
<td>28.3 ± 2.6</td>
<td>30.7 ± 1.6</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m.

The live weight at slaughter did not differ between treatment groups (Table 4.5). As percentages of live weight, subcutaneous adipose, visceral adipose and total adipose tissues, liver, empty carcass and testis weights did not differ between treatment groups (Table 4.5). The percentage of digestive tract weight to live weight and digestive tract length were greater in birds fed the high fat diet than in birds fed the low fat diet (p = 0.048 and p = 0.028 respectively) (Table 4.5).

Table 4.5: The body composition of male emus receiving either low or high fat diets for 54 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>low fat</th>
<th>high fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live weight at slaughter (kg)</td>
<td>42.6 ± 3.33</td>
<td>41.1 ± 2.02</td>
</tr>
<tr>
<td>Subcutaneous adipose (%)</td>
<td>19.2 ± 1.33</td>
<td>17.5 ± 1.26</td>
</tr>
<tr>
<td>Visceral adipose (%)</td>
<td>7.4 ± 0.43</td>
<td>7.0 ± 0.34</td>
</tr>
<tr>
<td>Total adipose (%)</td>
<td>26.6 ± 1.42</td>
<td>24.5 ± 1.52</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.05</td>
</tr>
<tr>
<td>Digestive tract weight (%)</td>
<td>1.5 ± 0.06 *</td>
<td>1.7 ± 0.11 *</td>
</tr>
<tr>
<td>Digestive tract length (%)</td>
<td>9.2 ± 0.28 *</td>
<td>11.2 ± 0.72 *</td>
</tr>
<tr>
<td>Testis (%)</td>
<td>0.014 ± 0.002</td>
<td>0.019 ± 0.003</td>
</tr>
<tr>
<td>Empty carcass (%)</td>
<td>52.3 ± 1.12</td>
<td>54.4 ± 1.53</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between treatment groups (p < 0.05). Parameters with a (%) next to them are given as the percentage tissue weight of live weight.
Hormones and metabolites

Serum glucagon did not differ between the high and low fat diet groups at any time point (Fig. 4.3). Within each group there was no difference in glucagon concentration over time.

Figure 4.3: Serum glucagon concentrations of male emus offered low (open circle) and high (solid circle) fat diets for 54 days. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m.
There was a trend for insulin to decrease over time in both groups. However, this was only significant for the high fat diet group ($p < 0.05$). The serum insulin concentrations were lower in the birds receiving the high fat than the low fat diet at Day 14, Day 28 and Day 42 ($p = 0.048$, $p = 0.016$ and $p = 0.048$ respectively) (Fig. 4.4).

**Figure 4.4:** Serum insulin concentration of male emus offered low (open circle) and high (solid circle) fat diets for 54 days. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m. An * indicates significant difference between treatment groups ($p < 0.05$).
In both groups it was observed that the insulin/glucagon ratio tended to decrease over time. However, this trend only achieved significance for the high fat diet group ($p < 0.05$) (Fig. 4.5). The insulin/glucagon ratio tended to be lower in the high fat diet group, however this was only significant on Day 28 ($p = 0.032$).

![Plasma insulin/glucagon ratio](image)

**Figure 4.5:** Serum insulin/glucagon ratio of male emus offered low (open circle) and high (solid circle) fat diets for 54 days. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m. An * indicates significant difference between treatment groups ($p < 0.05$).
The serum triiodothyronine concentrations did not differ between the groups receiving the high and low fat diets at any time point (Fig. 4.6). In both groups the triiodothyronine concentration tended initially to decrease, however this trend was not significant within either group over time.

Figure 4.6: Serum triiodothyronine concentration in male emus offered low (open circle) and high (solid circle) fat diets for 54 days. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m.
The serum thyroxine concentration was higher in the high fat diet group at Day 14 and lower at Day 42 (p = 0.048 and p = 0.028 respectively) (Fig. 4.7). Over time the thyroxine concentration decreased in both the low fat and the high fat diet groups (p < 0.05 and p < 0.01 respectively).

**Figure 4.7:** Serum thyroxine concentration in male emus offered low (open circle) and high (solid circle) fat diets for 54 days. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m. An * indicates significant difference between treatment groups (p < 0.05).
The triiodothyronine/thyroxine (T3/T4) ratio did not differ between the groups at any time point (Fig. 4.8). There was no difference over time within the group receiving the low fat diet. A difference in the T3/T4 ratio over time was observed within the group receiving the high fat diet (p < 0.05).

![Graph showing serum T3/T4 ratio in male emus offered low (open circle) and high (solid circle) fat diets for 54 days. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m.](image)

**Figure 4.8:** Serum T3/T4 ratio in male emus offered low (open circle) and high (solid circle) fat diets for 54 days. The shaded area represents the transition period of the high fat diet group from the low to the high fat diet. Values are means ± s.e.m.
4. Discussion
The results presented here do not support either of the hypotheses. Prolonged feeding of a high fat diet did not increase adiposity or appetite, or alter hormone concentrations to favour adipose storage (i.e. increased insulin, decreased glucagon, triiodothyronine and thyroxine). The absence of increases in adiposity and appetite, and of changes in hormone concentrations occurred despite more fat being consumed by the high fat diet group. As such, the absence of hormonal changes in these animals implies that in the emu this level of fat inclusion in the diet does not alter metabolism to favour energy storage. The failure of the high fat diet to increase appetite and adiposity may be due to the composition of the diet, taste aversions for components of the diet, or alterations in digestive physiology in response to the high fat diet restricting feed intake.
Alternatively, the higher energy content of the high fat diet may have caused a compensatory decrease in feed intake to maintain energy intake at a level appropriate to the animal's requirements.

A factor that may have contributed to the failure of the high fat diet to increase adiposity is its composition. First, the lower carbohydrate content of the high fat diet may have resulted in a greater proportion of the ingested fat being oxidised to meet the animal's energy requirements, particularly given the tendency for a lower level of energy intake in the high fat diet group. Second, there is evidence that the type of fat ingested can also affect the degree of adiposity. In rats it has been observed that feeding isoenergetic diets containing either saturated or unsaturated fat results in greater carcass adiposity in the saturated fat group (Shimomura et al, 1990). Broiler chickens receiving isoenergetic diets of either saturated or unsaturated fat exhibited greater abdominal adipose deposition in the group receiving saturated fat (Vilà & Esteve-Garcia, 1996; Sanz et al, 1999 & 2000). The difference between saturated fats and unsaturated fats in their ability to induce adiposity is most likely related to differences in their oxidation rates (Sanz et al, 2000). The greater the chain length of saturated fatty acids the slower their rate of oxidation, a situation favouring storage (Leyton et al, 1987). The diets formulated for this experiment used full-fat canola and soyabean meals as the primary sources of fat. As these fats are unsaturated, this may explain the lack of difference in adiposity between the two groups. In chickens, at high levels of inclusion, unsaturated fats, as fed in this experiment, increase adiposity (Vilà & Esteve-
Garcia, 1996). As such, the high fat diet may not have contained enough unsaturated fat to augment adipose deposition in emus.

An alteration in digestive function could also have been responsible for the lack of effect of the high fat diet. Diets that are high in fat slow the rate of stomach emptying (Mateos et al., 1982). It is therefore of interest that the weights and lengths of the gastrointestinal tract, as proportions of live weight, were higher in the group receiving the high fat diet. It is possible that this represents an adaptation to enable greater absorption of dietary components. A slower rate of stomach emptying may also explain the tendency for lower feed intake in the group receiving the high fat diet, as the animal would feel sated for longer due to stomach distension. A slower rate of stomach emptying in animals fed the high fat diet, and its satiating effects, may also explain the failure of the diet to increase adiposity. A satiated animal eats less and a tendency for lower energy intake was observed in the high fat diet group. As such, these animals would expend more energy absorbing components from the diet, and the energy obtained from this process is then more likely to be oxidised to meet the energetic requirements of the animal. This situation would not favour adipose deposition.

The concentrations of insulin (lower), glucagon, triiodothyronine and thyroxine (unchanged) also indicated that the metabolism of the birds fed the high fat diet did not favour adipose deposition. As such, the hypothesis that metabolic changes that favour adipose deposition will occur in response to a high fat diet can be rejected. However, the hormonal data provides other insights into the metabolism of the emu that may help to explain the failure of the high fat diet to increase adiposity. One of these explanations is that the emu staunchly resists manipulations that alter metabolism. The finding that significantly lower feed intake over a two week period was not associated with significant changes in hormone concentrations supports this explanation. The lower concentrations of insulin in the group receiving the high fat diet could mean that they were in negative energy balance. The birds receiving the high fat diet also consumed less food and had a lower energy intake, though neither was significant, that would have made a state of negative energy balance possible. In addition, at the final time-point the insulin concentration did not differ between the groups, following a two week period of rising feed intake in the high fat diet group, indicating that the energy balance had been restored in the high fat diet group. In contrast to insulin, glucagon did
not differ over the course of the experiment, implying that energy intake was sufficient. The elevated concentration of glucagon at Day 14 was most probably due to the decreased feed intake that occurred in response to the change in diet in the group receiving the high fat diet. The decrease in feed intake observed represents voluntary feed intake reduction in response to the presentation of an unfamiliar feed, a response also observed by other researchers working with emus (Van Cleeff, 2000 personal communication). Once accustomed to the new diet, feed intake was restored and the concentration of glucagon in serum became comparable to that of birds receiving the low fat diet. The insulin/glucagon ratio shows a strong tendency to be lower at all time points following exposure to the high fat diet. This indicates that metabolism has been altered to favour the utilisation of adipose stores rather than their deposition. The trends observed in both groups for increasing glucagon and decreasing insulin over the course of the experiment may reflect the onset of appetite suppression. This would suggest that high fat diets do not sustain appetite in emus, in contrast to observations in mice (Van Heek et al, 1997).

The rising feed intake of the group receiving the high fat diet during the final two weeks of the experiment may explain the lack of difference in insulin between the two groups at the final time point. The rising feed intake might also represent the induction of a mechanism to sustain appetite when energy intake preceding the reproductive period is insufficient. When the feed intake of emus is restricted over summer (maximal appetite) they respond by increasing their food intake and rate of weight gain in the following months (O’Malley, 1999). The increased food intake and weight gain occurs during a period when appetite would usually be declining. The ability to maintain their appetite when the preceding period has not satisfied their energetic requirements is a compensatory mechanism to ensure that the animals have sufficient reserves to survive the breeding period. The initial voluntary reduction in feed intake observed in response to the high fat diet and the lower feed intake while receiving the diet may have triggered such a mechanism. Normally after periods of voluntary feed intake reduction, such as occurs following stress in some birds (e.g. after handling), or after the completion of incubation, appetite returns rapidly and lost weight is quickly restored (Van Cleeff, 2001; Zadworny et al, 1985). I did not observe a rapid compensatory appetite following voluntary feed intake reduction in response to the high fat diet. This supports the idea
that the higher fat content decreased feed intake due to slower emptying of the stomach, or as a result of a taste aversion to the feed.

The circulating concentration of triiodothyronine was not different at any time-point. In both groups triiodothyronine concentrations initially decreased, and then increased from Day 14, with this tendency strongest in the group receiving the low fat diet. The slower rate of increase in the group receiving the high fat diet might reflect an effect of the high fat diet to decrease the metabolic rate. It may also result from a compensatory mechanism triggered by the voluntary starvation and lower energy intake of the group receiving the high fat diet. The thyroxine concentration was higher in the group receiving the high fat diet at Day 14 and lower at Day 42. The higher concentration of thyroxine at Day 14 in the group receiving the high fat diet may be due to the voluntary starvation observed in this group, as thyroxine is elevated in chickens in response to a 24 hour fast (Rosebrough & McMurtry, 2000). The lower concentration of thyroxine at the end of the experiment in the group receiving the high fat diet may reflect an effect of the high fat diet to decrease the metabolic rate. Thyroid hormone concentrations in emus and ostriches are highly variable (Dawson, 1996; Dawson et al, 1996; Blache et al, 2001c). In ostriches this variability is largely due to stress (Dawson & Deeming, 1997). The trends observed in triiodothyronine and thyroxine concentrations over the course of the experiment may, therefore, be artefacts of the handling procedures employed and the variability in thyroid hormone concentrations inherent in ratites. The absence of any differences between groups in the ratio of T3/T4 supports this idea.

In conclusion, dietary fat, at the degree of inclusion used in this experiment, does not increase adiposity, induce hyperphagia or cause a metabolic shift to favour adipose deposition. There is evidence that unsaturated fats, as used in this experiment, do not induce increases in adiposity as readily as saturated fats. If a higher level of fat inclusion, or saturated fats had been used, adiposity may have increased. The greater content of fat in the diet did cause changes in the digestive tract. These changes may have compensated for a slower rate of stomach emptying, as observed in other species receiving diets with a high fat content (Mateos et al, 1982). This slower rate of emptying and possibly a taste aversion to the high fat diet may be responsible for the lower feed intake and the lack of effect on adiposity in the group receiving this diet. Additionally, the difficulty encountered in changing emus from one diet to another may
have confounded the experimental results. Ideally the experiment should have been performed over a longer period. To overcome the limitations of dietary manipulations in emus more direct mechanisms of stimulating appetite and altering metabolism could be used. One such mechanism is treatment with glucocorticoids. In the following chapter I have investigated the effect of dexamethasone, a synthetic glucocorticoid, on appetite, adiposity and metabolism.
Chapter 5

The effect of dexamethasone on appetite, adiposity and the hypothalamic expression of NPY in female emus.

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1. Introduction

The decrease in appetite observed in emus during the breeding season leads to mobilisation of the adipose depot to meet energy demands (Blake, 1996; Blache & Martin, 1999; Blache et al, 2001b). It is probable that changes in appetite are centrally mediated, with the hypothalamus a logical site for investigation given its role in both satiety and appetite.

There are a number of pathways that could be involved in the control of appetite and adiposity in the emu. For example, pathways involving cocaine and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC), corticotrophin-releasing hormone (CRH), orexin/hypocretin and agouti-related protein (AGRP), are involved in the control of appetite and adiposity in other species (Mizuno et al, 1997; Ollmann et al, 1997; Sakurai et al, 1998; Heinrichs & Richard, 1999; Vrang et al, 1999). In this experiment I have focused on the principal pathway involving NPY as it is well characterised for a range of species including several avian species.

Neuropeptide Y (NPY) is a potent, centrally acting, appetite stimulant (Levine & Morley, 1984; Stanley & Leibowitz, 1984 & 1985; Stanley et al, 1985a & 1986; Kuenzel et al, 1987; White, 1993; Woods et al, 1998; Inui, 1999a). It is possible that mechanisms that disrupt appetite in the emu involve inhibition of NPY. The regulation of NPY synthesis and secretion occurs at the level of the hypothalamic-pituitary-adrenal (HPA) axis. This regulation occurs via changes in the concentration of insulin and glucocorticoids, which act antagonistically (Strack et al, 1995). The regulation of NPY by insulin and glucocorticoids is a potential mechanism for the long-term regulation of energy balance. As such, manipulating the hormonal balance could influence appetite indirectly and overcome NPY inhibition. The HPA axis is a logical target for modification. Manipulation of the HPA axis to alter the regulation of NPY could maintain appetite and adiposity when appetite is normally diminishing. This manipulation can be achieved with exogenous glucocorticoids, which diminish the ability of leptin to depress appetite, increase the expression of NPY mRNA and increase the concentration of insulin (Campbell et al, 1966; Simon, 1984; Mercer et al, 1996; Solano & Jacobson, 1999; Arvaniti et al, 1998; Zakrewska et al, 1997).
The action of glucocorticoids to first increase the expression of NPY mRNA, and second diminish leptin’s ability to increase appetite, promotes appetite. The elevated concentration of insulin in response to glucocorticoid treatment increases lipogenesis, gluconeogenesis and triglyceride deposition in adipose tissues. Increased appetite is not consistently observed in animals receiving exogenous glucocorticoids. The absence of increases in appetite may be due to the elevation of insulin inhibiting NPY mRNA expression, thereby removing the appetite stimulus (Schwartz et al., 1991; Dallman et al., 1993). However, increased adiposity occurs with glucocorticoid treatment even when appetite is not increased (Simon, 1984). As such, the elevated insulin concentrations, and their promotion of adipogenesis, are primarily responsible for the increased adiposity when appetite does not change in response to glucocorticoid administration.

A synthetic glucocorticoid, dexamethasone, induces many of the changes mentioned above in a wide range of species. In Syrian hamsters, chronic dexamethasone treatment increases NPY gene expression (Mercer et al., 1996). In rats, dexamethasone increases both synthesis and release of NPY (Corder et al., 1988). In sheep, injections of dexamethasone increase appetite and reduce weight loss in animals exported live to the Middle East (Adams & Sanders, 1992). Dexamethasone rapidly increases the concentration of insulin and leptin in humans, and increases the concentration of insulin in cattle (Corah et al., 1995; Kolaczynski et al., 1997; Miell et al., 1996; Papaspyrou-Rao et al., 1997). This leads to insulin resistance and favours adipose deposition. The elevation of leptin concentrations may also represent the induction of leptin resistance.

Glucocorticoid administration alters the metabolic rate. The administration of glucocorticoids decreases the circulating concentration of thyroxine, while having variable effects on the concentration of triiodothyronine (Darras et al., 1997). Depression of the thyroid hormones, and subsequently metabolic rate, may contribute to the increased adiposity observed in many species following dexamethasone treatment (Walker & Romsos, 1993).

Glucocorticoids also influence reproductive function. In chickens, acute dexamethasone treatment inhibits luteinising hormone secretion, rapidly reducing the circulating luteinising hormone concentration (Wilson & Lacassagne, 1978). If the
reduction in luteinising hormone concentration is sustained by chronic treatment it could act to maintain adiposity and appetite by removing the influence of reproductive hormones on metabolism and appetite.

An experiment was performed to determine if dexamethasone treatment, during a period when appetite is normally declining, enables appetite and adipose reserves to be sustained. The following hypotheses were formulated:

1. Dexamethasone administration will increase the mRNA expression of NPY,
2. Long-term dexamethasone administration will decrease the concentrations of triiodothyronine, thyroxine, and luteinising hormone, and increase the concentrations of blood glucose and insulin, and finally
3. Increased expression of NPY and metabolic changes induced by dexamethasone will be associated with increased appetite and adiposity.

To enable these hypotheses to be tested it was necessary to determine the correct dosage to use for emus. Therefore, a preliminary experiment was performed to determine the correct dosage of dexamethasone to use in the chronic trial. Dexamethasone treatment decreases the concentration of corticosterone to barely detectable levels (Buckland et al, 1974; Etches, 1976; Corder et al, 1988; Corah et al, 1995; Zakrzewska et al, 1999). As such, this can be used as an indicator of the onset and duration of the drug’s effects. The preliminary experiment is described in Section A. The long-term dexamethasone experiment is described in Section B.
Section A – Preliminary experiment

2A. Materials and Methods

Experimental design
To determine the correct dosage of dexamethasone to use in the long-term experiment male and female emus were treated with either a 6 mg or a 12 mg dose of dexamethasone. Blood samples were collected at regular intervals and the concentration of corticosterone was measured by radioimmunoassay to determine if the doses used were capable of suppressing corticosterone production and the duration of this effect.

Animals
Four male and four female emus that were approximately 12 months of age and hatched in the same season were used for this experiment. The experiment was performed during the non-breeding season. All the animals used for this experiment were in good health.

Treatment
Half of the birds of each sex were implanted with 6 mg of dexamethasone. The remaining birds were implanted with 12 mg of dexamethasone. The implants were placed subcutaneously in the area behind the wing. To do this a small incision was made in the skin and a purpose built tool was used to place the implant subcutaneously. The implants were not removed after completion of the experiment.

Blood sampling
As described in Chapter 2, 10 ml blood samples were collected. Blood samples were taken immediately before the implantation procedure, and again 7, 24, 72, 144, 240, 385 and 552 hours after implantation.

Hormones and metabolites
A radioimmunoassay was performed for corticosterone, as described in Chapter 2.

Data and statistical analysis
The significance of any differences between the 6 mg and 12 mg treated groups were determined at each time-point by Mann-Whitney U tests as described by Siegel (1956)
using the Statview\textsuperscript{TM} 512+ program (1986). This test was judged to be the most appropriate as samples were taken from two independent groups and the sample size was small \((n = 4, n = 4)\). The significance of differences over time was determined within groups by Friedman two-way analysis of variance by ranks as described by Siegel (1956). This test was judged to be the most appropriate as the samples were related and several time-points were under comparison. The results are indicated as the mean \(\pm s.e.m\).
3A. Results

*Hormones and metabolites*

The serum corticosterone concentration did not differ between the dosages tested at any time point. Over time both the 6 mg and 12 mg treated groups exhibited depression of their corticosterone concentration in response to the dexamethasone implant (p < 0.01 and p < 0.05 respectively). The corticosterone concentration began increasing again after 144 hours indicating the treatment duration was less than a week using implants of dexamethasone (Fig. 5.1).

![Graph showing serum corticosterone concentration over time](image_url)

**Figure 5.1:** The serum corticosterone concentration of emus treated with a single subcutaneous dexamethasone implants of either 6 mg (solid circle) or 12 mg (open circle). Values are means ± s.e.m.
4A. Discussion

The preliminary experiment indicated that a single dose of dexamethasone between 6 and 12 mg decreased the corticosterone concentration, although the levels remained detectable by the assay method employed. The results also indicate that using subcutaneous implants the treatment was effective for approximately one week. Trimedexil (dexamethasone trimethylacetate 5 mg/ml, Ilium Veterinary Products), was chosen for use in the long-term dexamethasone treatment experiment. Trimedexil has several advantages 1) it involves less handling stress as it is administered as a weekly injection rather than weekly subcutaneous implants, and 2) it is a long-lasting liquid preparation. This avoids problems relating to rapid clearance of the tablet form from the circulation and also differences in the rate of release of the implants between birds and over time. Both doses used in the preliminary experiment were equally capable of depressing corticosterone and it was therefore decided to use a dose that fell between those tested. As such, a dose of 10 mg/week was employed in the subsequent experiment. There was no apparent difference in response to corticosterone between males and females. Therefore, it was initially intended to include both males and females in the long-term trial. However, once the experiment had commenced, three birds in the male group were found to be females and another two died before the experiment was completed. As a result there were not enough males to give meaningful statistics and the male study group was abandoned. Prolonged treatment of catabolic doses of dexamethasone can cause muscle wasting. To be certain that dexamethasone is not catabolic when administered chronically at a dose of 10 mg/week, it is necessary to determine if muscle wasting occurs. In the long-term trial this was assessed by measuring the relative proportions of muscle and bone from a discrete region of the carcass.
Section B - Long-term dexamethasone treatment experiment

2B. Materials and methods

Experimental design
To test the hypotheses outlined in the introduction adult female emus were allocated into a control group receiving saline injections weekly or a treatment group receiving dexamethasone injections weekly. The mRNA expression of NPY was quantified in the mediobasal hypothalamus/preoptic area at the completion of the treatment period by ribonuclease protection assay. The concentration of triiodothyronine, thyroxine, luteinising hormone and insulin were measured at regular intervals over the course of the experiment by radioimmunoassay. Blood glucose was measured using a hand held glucometer. The association between changes in NPY, hormone and appetite and adiposity, were assessed by measuring food intake over the course of the experiment, weighing and fat scoring the animals at several time-points and performing carcass composition analysis at the completion of the experiment.

Animals
Twelve female emus, aged between one and two years were used. The birds were allocated into two groups of similar average weight. All birds were penned individually, with commercial emu feed available *ad libitum* (Glen Forrest Stockfeeders), see Chapter 2. The experiment commenced at the beginning of March, coinciding with the start of the breeding season.

Treatments
The groups comprised dexamethasone treated (*n* = 7) and saline treated (*n* = 5) animals. Treatments consisted of weekly injections of 10 mg of Trimedexil (dexamethasone trimethylacetate 5 mg/ml, Ilium Veterinary Products) for six weeks, given as a single 2 ml intramuscular injection. Saline treated birds experienced the same handling and received a 2 ml intramuscular injection of saline. The experiment was performed over a nine week period consisting of pretreatment (Day -14 through to Day 0) and treatment (Day 0 through to Day 49) periods.

Feed intake
Feed intake was recorded over the course of the experiment from Day -14. Recordings were carried out on three days per week to give a weekly feed intake value, as described
in Chapter 2.

**Live weight and fat scoring**

Birds were weighed at three time-points over the course of the experiment (Day -14, Day 14 and Day 49), immediately prior to bleeding and treatment. Fat scoring was performed twice, at the commencement and completion of the experiment as described in Chapter 2.

**Blood sampling**

As described in Chapter 2, 20 ml blood samples were collected weekly, immediately prior to treatment. An additional blood sample was collected from all birds on Day 2. After the blood glucose measurement, the remaining blood was dispensed into separate tubes for serum and plasma collection.

**Tissue collection**

At the completion of the experiment (Day 49), birds were bled and the blood glucose concentration was recorded prior to the animals being euthanased with Lethabarb as described in Chapter 2. The brain was removed and the hypothalamus and preoptic area collected for RNA extraction as described in Chapter 2.

**Carcass composition**

Carcass composition analysis was performed as described in Chapter 2. The weights of the subcutaneous adipose, visceral adipose and total adipose depots, and the liver, heart and ovary were recorded for all birds. To assess the degree of muscle catabolism after skinning and subcutaneous adipose tissue removal the total weight of the area between the hip and knee was measured for the left leg. The individual contributions of muscle and bone to this weight were then determined.

**Ribonuclease Protection Assay (RPA)**

RNA was extracted as described in Chapter 2. The ribonuclease protection assay was performed on total RNA extracted from the region containing the mediobasal hypothalamus and the preoptic area, using the RPA III kit (Ambion) as described in Chapter 2. The mRNA expression of NPY and β-actin were determined using the NIH Image program version 1.61. The sample number is one less for each group (ie
dexamethasone n=6, saline n=4), as a sample from each group was lost after collection.

**Hormones and metabolites**

Radioimmunoassay's were performed for corticosterone, insulin, triiodothyronine, thyroxine, and luteinising hormone, as described in Chapter 2. Immediately after each bleed, measurements were made of blood glucose using a hand held blood glucose meter as described in Chapter 2.

**Data and statistical analysis**

The expression of NPY was determined as a value relative to the expression of β-actin for each sample. The carcass composition data for each tissue was expressed as a percentage of the live weight of the animal. For the ovary, the value was expressed as a proportion of the ovary weight in grams, of the live weight in kilograms. Any effects of dexamethasone to promote muscle catabolism were assessed by determining the percentages of muscle and bone of the weight of the region between the hip and knee. The significance of differences at each time-point was determined by Mann-Whitney U tests as described by Siegel (1956) using the Statview™ 512+ program (1986). This test was judged to be the most appropriate as two independent groups were sampled from, the sample size was small (n1 ≤ 7, n2 ≤ 5) and the number of birds in each group was not equal. The significance of differences over time within groups was determined using Friedman two-way analysis of variance by ranks as described by Siegel (1956). This test was judged to be the most appropriate as the samples were related and more than two time-points were involved. The Chi-Square test of independence (McGrath, 1997) was used to determine the significance of any differences in fat score observed between groups at each time-point. This test was judged to be the most appropriate as the data consisted of frequencies in discrete categories, and two independent groups were under comparison. The Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) was used to determine the significance of any differences in fat score over time within each group. This test was judged to be the most appropriate as the samples were related and sample size was small (dexamethasone n = 4, saline n = 3). The results are indicated as the mean ± s.e.m.
3B. Results

Ribonuclease Protection Assay (RPA)

The expression of NPY mRNA did not differ between the dexamethasone treated (n = 6) and saline treated (n = 4) groups after 49 days of treatment (Fig. 5.2 and Table 5.1).

Table 5.1: The intensity of the mRNA expression of NPY relative to β-actin of female emus treated with either dexamethasone or saline for 49 days.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Dexamethasone (n = 6)</th>
<th>Saline (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY/β-actin</td>
<td>0.51 ± 0.054</td>
<td>0.54 ± 0.054</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m

Figure 5.2: The NPY mRNA expression of female emus treated with either dexamethasone (lanes 1-6) or saline (lane 7-10) for 49 days. Lane 11 and 12 contain the β-actin and NPY sense strand controls, respectively. Double-ended arrows indicate the position of the NPY (a) and β-actin (b) bands in the samples. Single-headed arrows indicate the position of the NPY (c) and β-actin (d) bands in the sense strand controls.
Chapter 5 – The effect of dexamethasone on appetite, adiposity and the hypothalamic expression of NPY in female emus.

Carcass composition

There was no difference in the weight of the subcutaneous or visceral adipose depots, total adiposity, the heart or the hip to knee region as a percentage of live weight. The weight of muscle and bone as a percentage of the hip to knee weight did not differ between the groups (Table 5.2). The weight of liver as a percentage of live weight was greater in the dexamethasone treated birds (p = 0.009). The proportion of ovary to live weight was lower in the dexamethasone treated birds (p = 0.009) (Table 5.2).

Table 5.2: The carcass composition of female emus treated with either dexamethasone or saline for 49 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dexamethasone (n = 7)</th>
<th>Saline (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous adipose (%)</td>
<td>9.3 ± 1.47</td>
<td>13.1 ± 1.98</td>
</tr>
<tr>
<td>Visceral adipose (%)</td>
<td>5.7 ± 0.73</td>
<td>5.5 ± 0.43</td>
</tr>
<tr>
<td>Total adipose (%)</td>
<td>15.0 ± 1.98</td>
<td>18.6 ± 2.35</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>2.18 ± 0.124*</td>
<td>1.35 ± 0.085*</td>
</tr>
<tr>
<td>Ovary (g/kg lwt)</td>
<td>0.28 ± 0.081*</td>
<td>3.70 ± 1.328*</td>
</tr>
<tr>
<td>Heart (%)</td>
<td>1.09 ± 0.059</td>
<td>1.00 ± 0.043</td>
</tr>
<tr>
<td>Hip to knee (%)</td>
<td>15.9 ± 0.74</td>
<td>15.4 ± 0.76</td>
</tr>
<tr>
<td>Muscle (% of hip to knee wt)</td>
<td>76.2 ± 2.62</td>
<td>80.5 ± 1.04</td>
</tr>
<tr>
<td>Bone (% of hip to knee wt)</td>
<td>20.7 ± 0.88</td>
<td>19.4 ± 1.05</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.009).
Feed intake

Feed intake was lower in the group treated with dexamethasone during Week 5 and Week 6 (p = 0.024 and p = 0.024 respectively) (Fig. 5.3). Within groups feed intake differed over time (a decreasing trend in the final three weeks) in the dexamethasone treated group (p < 0.01). Over time feed intake did not differ in the saline treated group.

Figure 5.3: Average daily feed intake per week of female emus treated with either saline (black bar) or dexamethasone (grey bar) for 49 days. Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.024).
Live weight and fat scores

Live weight did not differ between groups at Day -14 or Day 14. At Day 49 live weight was higher in the saline treated group (p = 0.024) (Fig. 5.4). Within groups the live weight increased over time in the saline treated group (p = 0.039). Over time live weight did not differ within the dexamethasone treated group.

Figure 5.4: Live weight of female emus treated with either saline (black bar) or dexamethasone (grey bar) for 49 days, on Day -14, Day 14 and Day 49. Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.024).
Chapter 5 – The effect of dexamethasone on appetite, adiposity and the hypothalamic expression of NPY in female emus.

The fat score decreased in the group treated with dexamethasone after 49 days of treatment ($p = 0.023$). The fat score showed a trend to increase in the group treated with saline after 49 days of treatment ($p = 0.05$). However, the fat scores did not differ on Day 0 or Day 49 between the treatment groups (Table 5.3).

**Table 5.3**: The fat scores of female emus on Day 0 and Day 49 of treatment with either dexamethasone or saline.

<table>
<thead>
<tr>
<th>Day</th>
<th>Dexamethasone (n = 7)</th>
<th>Saline (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.29 ± 0.474</td>
<td>2.80 ± 0.583</td>
</tr>
<tr>
<td>49</td>
<td>2.14 ± 0.634</td>
<td>4.00 ± 0.548</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m.
Chapter 5 – The effect of dexamethasone on appetite, adiposity and the hypothalamic expression of NPY in female emus.

Hormones and metabolites

The serum insulin concentration did not differ between or within treatment groups at any time point (Fig. 5.5).

![Graph showing serum insulin concentration over time.

**Figure 5.5**: Serum insulin concentration of female emus treated with either saline (open circle) or dexamethasone (solid circle) for 49 days. Values are means ± s.e.m.
The blood glucose concentration did not differ between the groups before treatment commenced (Day -14, Day -7 and Day 0). Following the commencement of dexamethasone treatment (Day 0), the blood glucose concentration was higher in the group treated with dexamethasone (p < 0.003) (Fig. 5.6). Over time the blood glucose concentration increased in the group treated with dexamethasone (p < 0.001). Over time the blood glucose concentration decreased in the group treated with saline (p < 0.01).

Figure 5.6: Blood glucose concentration of female emus treated with either saline (open circle) or dexamethasone (solid circle) for 49 days. Values are means ± s.e.m. An * indicates significant difference between groups (p < 0.01).
The serum thyroxine concentration was lower in the group treated with dexamethasone than the group treated with saline from Day 7 until the completion of the experiment ($p = 0.001$) (Fig 5.7). Within groups thyroxine decreased over time in the dexamethasone, but not the saline treated group ($p < 0.001$).

**Figure 5.7:** Serum thyroxine concentration of female emus treated with either saline (open circle) or dexamethasone (solid circle) for 49 days. Values are means ± s.e.m. An * indicates significant difference between groups ($p = 0.001$).
The serum triiodothyronine concentration was higher in the dexamethasone than the saline treated group on Day 7 and Day 14 (p = 0.009 and p = 0.015 respectively) (Fig. 5.8). Within the dexamethasone treated group triiodothyronine differed over time (p < 0.02). Over time there was no difference within the saline treated group.

Figure 5.8: Serum triiodothyronine concentration of female emus treated with either saline (open circle) or dexamethasone (solid circle) for 49 days. Values are means ± s.e.m. An * indicates significant difference between groups (p < 0.05).
The triiodothyronine/thyroxine (T3/T4) ratio increased over time in the group treated with dexamethasone, but did not change within the group treated with saline (p < 0.001). The T3/T4 ratio was higher in the group treated with dexamethasone than the group treated with saline from Day 2 until the completion of the experiment (p < 0.05) (Fig. 5.9).

**Figure 5.9:** T3/T4 ratio of female emus treated with either saline (open circle) or dexamethasone (solid circle) for 49 days. Values are means ± s.e.m. An * indicates significant difference between groups (p < 0.05).
The serum luteinising hormone concentration was higher in the group treated with dexamethasone than the group treated with saline at Day 14 and Day 21 (p = 0.037 and p = 0.001 respectively) (Fig. 5.10). There was no difference in the luteinising hormone concentration over time within either group.

Figure 5.10: Serum luteinising hormone concentration of female emus treated with either saline (open circle) or dexamethasone (solid circle) for 49 days. Values are means ± s.e.m. An * indicates significant difference between groups (p < 0.05).
The serum corticosterone concentration of the group treated with dexamethasone was higher at Day -14, and lower from Day 2 until the completion of the experiment (p = 0.04 and p < 0.02 respectively) (Fig. 5.11). Within groups the corticosterone concentration decreased over time in the group treated with dexamethasone, but did not change in the group treated with saline (p < 0.001).

**Figure 5.11**: Serum corticosterone concentration of female emus treated with either saline (open circle) or dexamethasone (solid circle) for 49 days. Values are means ± s.e.m. An * indicates significant difference between groups (p < 0.05).
4B. Discussion

The results presented here do not support the hypotheses that prolonged treatment with dexamethasone increases NPY gene expression, or is associated with increased appetite and adiposity. The hypothesis that dexamethasone treatment would change the concentration of metabolic hormones is partially supported by the results, with thyroxine decreased and blood glucose increased as predicted. The remaining hormones measured did not change in the manner hypothesised. As NPY is a potent appetite stimulant the failure of dexamethasone to increase NPY gene expression is most likely responsible for the lack of increase in appetite. The failure to increase adiposity may be due to the absence of both increased appetite and increased insulin concentrations, and therefore adipogenesis. This implies that in the emu dexamethasone treatment does not induce changes that facilitate increases in appetite or adiposity. As such, long-term dexamethasone treatment is not a viable option for maintaining or increasing adiposity in emus during periods when adipose stores are mobilised.

Dexamethasone treatment also failed to depress triiodothyronine or luteinising hormone. This could indicate that the dose used was too low. Muscle catabolism was not observed following dexamethasone treatment, indicating that the dose of dexamethasone given did not induce a catabolic state as is observed when administered at high doses. The lower concentrations of corticosterone in the dexamethasone treated birds from Day 2, indicates that the dosage did suppress the adrenocortical axis. It must be noted that the significantly higher concentration of corticosterone prior to the commencement of treatment (Day -14 and Day -7) in the dexamethasone treated group resulted from the allocation process to obtain similar starting live-weights in both groups. Three of the birds allocated to the dexamethasone treatment group were extremely stressed by handling resulting in their corticosterone concentrations being very high, and raising the group average. The thyroxine concentration was decreased and the blood glucose concentration increased by dexamethasone treatment, as hypothesized. However, as the thyroxine concentration decreased without increasing adiposity it can be argued that decreased thyroxine was not associated with depression of metabolic rate. The absence of any differences between the groups in their triiodothyronine concentrations and adiposity despite significantly decreased thyroxine and an increased T3/T4 ratio in the dexamethasone treated group indicates that
triiodothyronine may be more important than thyroxine in controlling metabolism in the emu.

There was a tendency for the total and subcutaneous adiposity to be lower in the dexamethasone treated group possibly due to the decreased feed intake observed in this group over the final two weeks of the experiment. Dexamethasone treatment resulted in an increased liver percentage of live-weight, most likely due to lipid infiltration. Finally dexamethasone treatment was associated with suppression of ovarian development. The depression of ovarian development may not have been a direct effect, but rather a consequence of the suppressed appetite, weight loss and decreasing adiposity during the final stage of the experiment in the dexamethasone treated group. This is supported by the lack of any sustained differences in luteinising hormone concentrations between the dexamethasone and saline treated groups over the course of the experiment.

These results have two implications. First, that under these conditions NPY does not play a major role in the control of appetite in the emu, as diminished appetite in the dexamethasone treated birds at the completion of the experiment was not associated with decreased NPY gene expression. Indeed, it has been illustrated in mice that multiple redundant pathways exist that control appetite in the absence of NPY (Erickson et al, 1996). Therefore, it is possible that genes other than NPY could be of greater importance to the control of appetite in emus under these conditions. Genes that could be of greater importance than NPY to the control of appetite in the emu include orexin/hypocretin, proopiomelanocortin (POMC), agouti-related protein (AGRP), cocaine and amphetamine-regulated transcript (CART) and corticotrophin-releasing hormone (CRH). Second, as this experiment was performed during the period where appetite is decreasing at the commencement of the breeding season, it is possible that some breeding season or photoperiod-related factor was inhibiting the response of NPY to dexamethasone and also possibly its action to stimulate appetite.

There is evidence that the responsiveness of appetite to stimulating factors changes seasonally in birds. One such factor that increases appetite via NPY is prolactin. In non-breeding ring doves prolactin increases appetite (Buntin & Figge, 1988; Hnasko & Buntin, 1993; Buntin et al, 1999). In the ring dove prolactin increases appetite by
increasing NPY-immunoreactive cell bodies in the infundibular region of the hypothalamus (Strader & Buntin, 2001). In contrast, prolactin administration to turkeys during the reproductive period either decreases or has no effect on appetite (Denbow, 1986). As such, the diminished appetite that is observed during the breeding season in the emu could indicate inhibition of either NPY’s response to prolactin, or of NPY’s ability to increase appetite. The inability of dexamethasone to increase NPY mRNA gene expression during this experiment could also be due to inhibition of NPY’s response to appetite stimulants via the action of photoperiod-related factors. The potential for factors to inhibit NPY’s response to appetite stimulants during the breeding season will be investigated in the following chapter. I will investigate NPY expression during incubation in the male emu, a model that is characterized by elevated prolactin concentrations.
Chapter 6

Changes in food intake, adiposity and the hypothalamic expression of NPY and VIP in response to incubation.
Chapter 6 – Changes in food intake, adiposity and the hypothalamic expression of NPY and VIP in response to incubation

1. Introduction

Incubating male emus exhibit a much greater reduction in appetite and adiposity than their non-incubating counterparts or females during the breeding season (Blake, 1996). The decreased appetite of the incubating male is a form of anorexia. Naturally occurring anorexia is observed in many species during activities such as, hibernation, incubation and migration. This state of voluntary fasting occurs via a “sliding set point” for energy reserves that allows the animal to maintain a depressed appetite, despite decreasing energy reserves, during periods when appetite would hinder more important activities (Mrosovsky & Sherry, 1980).

In almost all species studied, food restriction or starvation increases NPY gene expression and corticosterone concentrations (Boswell et al, 1999; McShane et al, 1993; Chua et al, 1991; Dallman et al, 1999). In humans, increased NPY gene expression is also observed during anorexia nervosa (Støving et al, 1999). There are a number of pathways that could be involved in the control of appetite during incubation in the emu. For example, pathways involving cocaine and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC), corticotrophin-releasing hormone (CRH), orexin/hypocretin and agouti-related protein (AGRP), are involved in the control of appetite and adiposity in other species (Mizuno et al, 1997; Ollmann et al, 1997; Sakurai et al, 1998; Heinrichs & Richard, 1999; Vrang et al, 1999). In this experiment it was decided to focus on the principal pathway involving NPY as it is well characterised for a range of species including several avian species. However, the expression of NPY mRNA during the period of voluntary starvation, which accompanies incubation in many avian species, has not been investigated.

The onset of incubation behaviour in avian species is triggered by a rise in vasoactive intestinal polypeptide (VIP) (Sharp et al, 1989). VIP stimulates prolactin release, inducing and maintaining incubation behaviour (Chaiseha & El Halawani, 1999; El Halawani et al, 1996; Sharp et al, 1989). Prolactin has been shown to affect appetite, but its effects may be dependent upon reproductive status. Prolactin increases appetite in ring doves (non-breeding), and decreases appetite in laying turkeys (Denbow, 1986; Buntin & Figge, 1988; Hnasko & Buntin, 1993; Buntin et al, 1999). In non-breeding ring doves, the central administration of prolactin increases the number of NPY-immunoreactive cell bodies
indicating that the stimulation of appetite by prolactin is mediated at least partially by NPY (Strader & Buntin, 2001). During the breeding season, prolactin may be prevented from affecting appetite by photoperiod-related factors. These photoperiod-related factors could act to prevent prolactin's actions in several ways. First, in contrast to the non-breeding birds, NPY gene expression may not increase during the breeding period in response to elevated prolactin concentrations (Strader & Buntin, 2001). Second, NPY gene expression might increase in response to elevated prolactin concentrations, but its ability to increase appetite inhibited by other factors generated by the metabolic changes that promote energy sparing mechanisms during incubation. One such factor could be corticotrophin-releasing hormone (CRH). CRH acts centrally to inhibit the effects of NPY on appetite (McCarthy et al, 1993; Menzaghi et al, 1993). CRH also stimulates adrenocorticotrophin (ACTH) secretion, and this stimulates the release of glucocorticoids from the adrenal gland (Kacsoh, 2000). Experimental treatments that increase CRH immunoreactivity or gene expression in the hypothalamus, and the administration of CRH receptor agonists or CRH, are associated with increased concentrations of corticosterone (Gupta & Brush, 1998; Grill et al, 2000; Bruijnzeel et al, 2001; Helmreich et al, 2001).

Metabolic changes may also be directly involved in the control of appetite during incubation. In incubating birds increased glucagon and decreased insulin concentrations promote lipolysis (Chieri et al, 1972; Cherel et al, 1988). The lipid fuels supplied by the catabolism of adipose reserves decrease the utilisation of glucose and maintain a constant blood glucose concentration (Groscolas & Rodriguez, 1981; Le Maho et al, 1981). Incubation is also associated with lower triiodothyronine and thyroxine concentrations, which decrease metabolic rate and thereby spare energy reserves (Cherel et al, 1988; Groscolas & Leloup, 1989). In emus, incubation is associated with increased glucagon and decreased insulin and thyroxine concentrations (Van Cleeffe, 2001). As such, the hormonal balance of the incubating emu is consistent with a catabolic state. However, comparisons of hormone concentrations between non-incubating and incubating male emus during the breeding season have not been made. Also, differences in hormone concentrations between non-incubating and incubating male emus may partially explain the greater degree of appetite suppression in the incubating emu.

An experiment was designed to identify associations between metabolic hormone
concentrations and neuropeptides at the hypothalamic level, and the decrease in appetite during incubation. The following hypotheses were formulated:

1. NPY gene expression in the hypothalamus will be higher in incubating than in non-incubating male emus,

2. Hypothalamic VIP gene expression and prolactin concentration will be higher in incubating than in non-incubating male emus,

3. Hormonal changes consistent with a catabolic state (increased glucagon and decreased insulin concentrations), and reduced resting metabolic rate (decreased thyroxine and decreased or unchanged triiodothyronine concentrations) will be observed in incubating emus, with blood glucose levels maintained at a constant level, and finally,

4. The concentration of corticosterone will be higher in the incubating than the non-incubating male emus.
2. Materials and Methods

Experimental design

To test the hypotheses outlined above male emus were allocated into two groups, one group was given eggs and allowed to incubate for 6 weeks. The second group received no visual or tactile contact with eggs and constituted the control group. At the completion of the experiment the region containing the mediobasal hypothalamus/preoptic area was collected and RNA was extracted. The expression of mRNA for NPY and VIP in the mediobasal hypothalamus/preoptic area were quantified by ribonuclease protection assay. In addition food intake was measured over the course of the experiment to determine if any associations existed with the expression of NPY mRNA. The concentration of prolactin, insulin, glucagon, thyroxine, triiodothyronine and corticosterone were measured over the course of the experiment by radioimmunoassay. The blood glucose concentration was measured using a glucometer. In addition, carcass composition analysis was performed to determine the extent of catabolism of adipose and selected muscle tissue.

Animals

Eleven sexually mature male emus were penned individually and given *ad libitum* access to commercial breeder pellets (Glen Forrest Stockfeeds) as described in Chapter 2.

Treatments

From June, five birds from the incubating group were given an egg every two to three days until they had nine eggs or commenced incubation. The eggs given to the birds were from the previous breeding season and had been blown and refilled with sand to their original weight to ensure that enough eggs were available for the experiment and prevent problems with them going rotten. The eggs were then painted with a non-odorous paint approximating the original shell colour to account for any leaching of colour that had occurred. The birds were allowed to incubate for six weeks. A group of six birds were used as the controls.

Feed intake

Feed intake was determined during Week 6 of incubation. A control bird was matched to each incubator for feed intake measurement. The birds were slaughtered together at the end of Week 6 of incubation so that effects of changing photoperiod on feed intake
between paired individuals were accounted for and differences in appetite observed could be attributed to reproductive status.

**Blood sampling**
Once a week from Week 3 of incubation a 20 ml blood sample was collected, as described in Chapter 2, from the incubator and its matched control.

**Tissue collection**
Following euthanasia the mediobasal hypothalamus and the preoptic area were excised as described in Chapter 2 for RNA extraction and RPA analysis.

**Carcass composition**
The carcass composition was carried out as described in Chapter 2. The gastrocnemius lateralis from the left leg of each bird was also dissected out and weighed to determine if protein catabolism contributed to the loss of live weight observed in incubating birds.

**RPA analysis**
RNA was extracted as described in Chapter 2. The RPA was performed on total RNA extracted from the region containing the mediobasal hypothalamus and the preoptic area, using the RPA III kit (Ambion) as described in Chapter 2. The mRNA expression of NPY, VIP and β-actin were determined by measuring the intensity of the bands for each gene visualised on a 1 x TBE, 5% acrylamide gel. The band intensity was determined using the NIH Image program version 1.61.

**Hormones and metabolites**
Radioimmunoassays were performed for insulin, triiodothyronine, thyroxine, prolactin, corticosterone and glucagon for all samples as described in Chapter 2. Immediately after each bleed measurements were made of blood glucose using a hand held blood glucose meter (Precision Q.I.D, Medisense) as described in Chapter 2.

**Data and statistical analysis**
The expression of VIP and NPY were determined as values relative to the expression of β-actin for each sample. For the carcass composition analyses each tissue weight was
converted to a percentage of the live weight of the animal. The significance of any
differences observed between incubating and non-incubating male emus was determined at
each time-point using Mann-Whitney U tests as described by Siegel (1956) using the
Statview™ 512+ program (1986). The significance of trends over time within each group
was determined using the Friedman two-way analysis of variance by ranks as described by
Siegel (1956). Non-parametric statistical analysis was judged to be the most appropriate as
two independent groups were sampled from, the sample size was small, and the number of
birds in each group was not equal. The results are indicated as the mean ± s.e.m.
3. Results

RPA

The level of VIP mRNA expression was higher in the incubating males and they also displayed a trend for elevated NPY mRNA expression (p = 0.026 and p = 0.165 respectively) (Fig. 6.1 and Table 6.1).

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Incubators</th>
<th>Non-incubators</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY/β-actin</td>
<td>0.69 ± 0.046*</td>
<td>0.53 ± 0.083</td>
</tr>
<tr>
<td>VIP/β-actin</td>
<td>0.62 ± 0.046*</td>
<td>0.47 ± 0.055</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.026).

Figure 6.1: The NPY and VIP mRNA expression of incubating (lanes 1-5) and non-incubating (lanes 6-11) male emus determined using the RPA technique. Lanes 12, 13 and 14 contain respectively, the sense strand positive controls for emu β-actin, NPY and VIP. Arrows indicate the position of the bands for VIP (a, d), NPY (b, e) and β-actin (c, f) from samples and sense strand controls respectively.
**Food intake, carcass composition and live weight**

Food intake, live weight and percentage total fat, subcutaneous fat and visceral fat weights of live weight and the percentage liver weight of live weight were all lower in the incubating group (p = 0.002, p = 0.015, p = 0.009, p = 0.015, p = 0.041 and p = 0.041 respectively) (Table 6.2). The percentage of the gastrocnemius lateralis muscle of live weight was higher in the incubating group (p = 0.026) (Table 6.2).

**Table 6.2:** The total feed intake over Week 6 of incubation and the body composition of incubating and non-incubating male emus at the end of Week 6 of incubation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Incubators</th>
<th>Non-incubators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total feed intake (kg)</td>
<td>0.20 ± 0.148*</td>
<td>6.93 ± 1.581*</td>
</tr>
<tr>
<td>Live weight (kg)</td>
<td>35.9 ± 1.95*</td>
<td>41.8 ± 1.03*</td>
</tr>
<tr>
<td>Subcutaneous adipose (%)</td>
<td>11.7 ± 2.14*</td>
<td>17.6 ± 1.11*</td>
</tr>
<tr>
<td>Visceral adipose (%)</td>
<td>4.09 ± 0.889*</td>
<td>5.93 ± 0.518*</td>
</tr>
<tr>
<td>Total adipose (%)</td>
<td>15.8 ± 2.91*</td>
<td>23.5 ± 1.24*</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>1.17 ± 0.152*</td>
<td>1.74 ± 0.152*</td>
</tr>
<tr>
<td>Gastrocnemius lateralis muscle (%)</td>
<td>1.53 ± 0.065*</td>
<td>1.37 ± 0.042*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p < 0.05).
**Hormones and metabolites**

The serum prolactin concentration was higher in the incubating males during Week 3, Week 4, Week 5 and Week 6 ($p = 0.004$, $p = 0.048$, $p = 0.002$ and $p = 0.002$ respectively). Within groups there were no difference in prolactin concentration over time (Fig. 6.2).

**Figure 6.2:** The serum prolactin concentration from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m. An * indicates significant difference between groups ($p < 0.05$).
The blood glucose concentration did not differ between or within either group at any time point (Fig. 6.3).

**Figure 6.3:** Mean blood glucose concentrations from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m.
There was a tendency for the serum glucagon concentration to be higher at all time points in the incubating group. Serum glucagon concentrations were higher in the incubating group, at Week 4 and Week 5 of incubation ($p = 0.028$ and $p = 0.032$ respectively). Within groups glucagon concentrations did not differ over time (Fig. 6.4).

**Figure 6.4:** Mean serum glucagon concentrations from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m. An * indicates significant difference between groups ($p < 0.05$).
The serum insulin concentration did not differ between or within groups at any time point (Fig. 6.5).

Figure 6.5: Mean serum insulin concentrations from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m.
The insulin/glucagon ratio was higher in the non-incubating males at Week 4 and Week 5 of incubation (p = 0.028 and p = 0.032 respectively). There was also a tendency for the ratio to be higher in the non-incubating males at Week 3 (p = 0.057). Within groups the ratio did not differ over time for either group (Fig. 6.6).

**Figure 6.6**: Mean serum insulin/glucagon ratio from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m. An * indicates significant difference between groups (p < 0.05).
The serum triiodothyronine concentration did not differ between or within groups at any time point (Fig 6.7).

Figure 6.7: Mean serum triiodothyronine concentrations from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m.
The serum thyroxine concentration was higher in the non-incubating males at each sampling point ($p = 0.004$) (Fig. 6.8). Within groups there was an effect of time in the incubating group ($p < 0.05$). Over time the thyroxine concentration did not change in the non-incubating group.

**Figure 6.8:** Mean serum thyroxine concentrations from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m. An * indicates significant difference between groups ($p < 0.05$).
The triiodothyronine/thyroxine (T3/T4) ratio did not differ between or within groups at any time point (Fig. 6.9).

Figure 6.9: The T3/T4 ratio from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m.
The serum corticosterone concentration tended to be higher at all time points in the incubating emus (Fig. 6.10). This trend was significant at Week 3 and Week 6 ($p = 0.028$ and $p = 0.048$ respectively). Within groups the concentration of corticosterone did not differ over time for either group.

**Figure 6.10**: Mean serum corticosterone concentrations from Week 3 to Week 6 of incubation of incubating (solid circle) and non-incubating (open circle) male emus. Values are means ± s.e.m. An * indicates significant difference between groups ($p < 0.05$).
4. Discussion

The hypothesis that hypothalamic NPY gene expression is higher in incubating than non-incubating emus is not supported by the results, although there was a tendency for higher levels of expression in the incubating group. The results support the hypothesis that hypothalamic VIP expression and circulating prolactin levels are increased in incubating emus. This suggests that as with other avian species, in emus, VIP and prolactin are involved in the maintenance of incubation behaviour (Mauro et al., 1989; Sharp et al., 1989). The hypothesis that emus enter a catabolic state during incubation, with increased glucagon and thyroxine concentrations and a tendency for decreased insulin concentrations is supported by the results. The hypothesis that corticosterone concentration is higher in incubating emus is also supported, with a tendency for higher concentrations in the incubators throughout the study period and significantly higher concentrations at two of these time points.

The absence of a significant difference in NPY expression between incubating and non-incubating emus during the breeding season raises some interesting possibilities. First, NPY expression may be at high levels in both incubators and non-incubators during the breeding period due to diminishing adipose reserves. If NPY expression is high, the diminished appetite during the breeding period is probably the result of inhibition of appetite promotion by NPY. A candidate for the role of NPY inhibitor is corticotrophin-releasing hormone (CRH) a centrally acting hormone that prevents NPY increasing appetite (McCarty et al., 1993; Menzaghi et al., 1993). The higher concentration of corticosterone in the incubating birds lends support to a role for CRH, as CRH administration, increased CRH expression or immunoreactivity, and CRH receptor stimulation are associated with increased corticosterone concentration (Gupta & Brush, 1998; Grill et al., 2000; Bruijnzeel et al., 2001; Helmreich et al., 2001). Alternatively, the higher concentration of corticosterone in the incubating emus in this experiment may reflect greater stress due to the animals being in negative energy balance. Second, the ability of NPY to respond to decreasing adipose reserves may be inhibited during the breeding season. Higher concentrations of prolactin in the incubating birds were not associated with elevations in the level of NPY mRNA expression. This result is in contrast to the finding that NPY was elevated in ring doves administered prolactin during the non-breeding period (Strader & Buntin, 2001). The absence of significant differences in NPY
mRNA expression supports the idea that NPY's ability to respond to factors, such as prolactin, that would normally stimulate NPY expression and thereby appetite, could be inhibited during the breeding season. A final possibility is that NPY may not have a pivotal role in the regulation of body reserves in the emu. Appetite in emus might be controlled at the hypothalamic level by other appetite mediators, such as, orexin, proopiomelanocortin (POMC), agouti-related protein (AGRP), CRH or cocaine and amphetamine-regulated transcript (CART).

As expected the anorexia and subsequent negative energy balance during incubation led to catabolism of adipose and liver energy reserves to meet the animals energetic requirements, resulting in lower proportions of adipose and liver tissue to live weight. The catabolic state did not include depletion of muscle glycogen or protein as evidenced by the higher proportion of the gastrocnemius muscle to live weight in the incubating birds. This does not mean that muscle was increased during incubation, rather that muscle mass was unchanged and subsequently increased as a proportion of live weight as the adipose stores and live weight decreased.

The hormonal data also supports a catabolic state in the incubators whose glucagon concentrations were higher, insulin concentrations tended to be lower and ratio of insulin to glucagon was lower. As expected these changes in insulin and glucagon maintained a constant level of blood glucose that did not differ from that of the non-incubating birds. The expectation that the decreased activity and feed intake during incubation would lower the metabolic rate is supported by the lower concentration of thyroxine in the incubators. However, triiodothyronine and the T3/T4 ratio were not different between the two groups. Overall these results suggest that incubation in the emu is associated with a decrease in the metabolic rate and catabolism of adipose reserves. Other researchers have also observed a decreased metabolic rate in the emu during incubation (Buttemer & Dawson, 1989a & 1989b).

As *ad libitum* food was available, the low level of intake of the non-incubating, and the anorexia of the incubating birds, resulted from depressed appetite. In the following chapter I examined the response of NPY and VIP mRNA expression and the concentration of metabolic hormones to short-term starvation in order to obtain a better understanding of
the control of appetite and adiposity in the emu. Imposed starvation induces many of the alterations in metabolic hormones that are observed during anorexia. However, imposed starvation is more stressful than naturally occurring anorexia and differs significantly in that it is associated with increased appetite. The increase in appetite is mediated by increased NPY expression and changes in metabolic hormones. As such, investigating appetite and adiposity using the model of short-term starvation will provide further insights into the control of appetite and adiposity in the emu.
Chapter 7

The effect of short-term starvation on the hypothalamic expression of NPY and VIP during a period of elevated appetite.

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<th>Section</th>
<th>Page</th>
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</thead>
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</tbody>
</table>
Chapter 7 - The effect of short-term starvation on the hypothalamic, expression of NPY and VIP during a period of elevated appetite

1. Introduction
Throughout the course of the year emus undergo large shifts in their food intake, body weight and metabolism. These changes include anorexia in incubating males coupled with extensive utilisation of the adipose stores. Following the cessation of the breeding period appetite is up-regulated and fat is deposited.

In Chapter 6, incubation was associated with increased expression of VIP mRNA in the mediobasal hypothalamus/preoptic area, and a tendency towards elevated NPY mRNA expression. In Chapter 5, NPY mRNA expression was unchanged in the same region by dexamethasone treatment despite decreased appetite and a tendency for lower levels of adiposity in the treated birds. As Chapters 5 and 6 investigated birds during the breeding season, the actions and responses of NPY to decreasing adiposity may have been suppressed by factors associated with reproduction and photoperiod.

There are a number of pathways that could be involved in the control of appetite and adiposity in the emu. For example, pathways involving cocaine and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC), corticotrophin-releasing hormone (CRH), orexin/hypocretin and agouti-related protein (AGRP), are involved in the control of appetite and adiposity in other species (Mizuno et al, 1997; Ollmann et al, 1997; Sakurai et al, 1998; Heinrichs & Richard, 1999; Vrang et al, 1999). In this experiment it was decided to focus on the principal pathway involving NPY as it is well characterised for a range of species including several avian species. To clarify the role of NPY, and investigate if VIP has a role in the control of appetite in emus, it is necessary to determine how they respond to imposed starvation when appetite is maximal. In chickens, NPY mRNA expression is increased by starvation or feed restriction (Boswell et al, 1999). The effects of starvation on VIP at the brain level have not been widely studied. However, in rats the concentration of VIP in the brain is not altered by starvation (Shulkes et al, 1983).

Starvation, in addition to its central effects, alters the metabolic status of the animal. When energy intake is insufficient animals enter a catabolic state that, in birds, is characterised by decreased insulin and increased glucagon (Sitbon & Mialhe, 1978; Rosebrough et al, 1984). Insufficient energy intake leads to the catabolism of adipose and protein reserves to meet the animals continuing energy requirements. Catabolism of adipose tissue increases the concentration of β-hydroxybutyrate, while protein breakdown increases the
concentration of urea (Le Maho et al, 1981; García-Rodríguez et al, 1987). In chickens, a two day fast decreases serum triiodothyronine and increases serum thyroxine concentrations (Van der Geyten et al, 1999). Corticosterone concentrations are also elevated by starvation and are associated with increases in NPY expression (Dallman et al, 1999; Geris et al, 1999). In addition, adrenalectomy of rats decreases NPY elicited food intake by 60-71% and this is returned to unoperated levels by corticosterone replacement (Stanley et al, 1989). It can be inferred from this that corticosterone is required for exogenous NPY to affect appetite. The rise in NPY expression in response to starvation follows the increase in corticosterone (Dallman et al, 1999). As such, increased corticosterone may be a requirement for increases in NPY expression or for potentiation of its effects on appetite.

This experiment was performed to determine how short-term starvation during a period of elevated appetite affects metabolism and the mRNA expression of NPY and VIP. The following hypotheses were tested:

1. Starvation, when appetite is high, will increase the expression of NPY mRNA without affecting VIP mRNA expression in the mediobasal hypothalamus/preoptic area,

2. That starvation would alter the concentration of hormones and metabolites to favour catabolism of adipose tissue in the starved birds (concentrations of glucagon, β-hydroxybutyrate and thyroxine will increase and insulin and triiodothyronine will decrease). As the starvation period is short, catabolism of protein is not expected to be initiated, therefore the concentration of urea will not change, and finally

3. Elevated corticosterone concentrations will be observed in response to starvation.
2. Materials and Methods

Experimental design

To test the hypotheses outlined above male and female emus were allocated into two groups. The first group was used as a control, while the second group was subjected to a one week period of starvation. At the completion of the starvation period the region containing the mediobasal hypothalamus/preoptic area was collected and RNA was extracted. The expression of NPY and VIP mRNA in the mediobasal hypothalamus/preoptic area was quantified by ribonuclease protection assay. The concentrations of insulin, glucagon, thyroxine, triiodothyronine and corticosterone were measured before and at the completion of the treatment period by radioimmunoassay. The concentration of urea and β-hydroxybutyrate were measured before and at the completion of the treatment period by enzymatic and spectrophotometric assays respectively. In addition, the birds were weighed before, and at the completion of the treatment period, and carcass composition analysis was performed on a subset of birds to determine the extent to which the adipose reserves were catabolized (Fig. 7.1).

Animals

A group of sixteen emus (seven males and nine females) were divided into two groups comprising eight birds starved (five female and three male) and eight control birds (four female and four male). All animals were kept in individual pens.

Treatment

The experiment commenced mid-November, a period when appetite is maximal. All the birds were given ad libitum access to a commercial pellet feed (Glen Forrest Stockfeeders)
for one week. The feed was then removed from the eight animals in the starved group for a period of seven days.

**Measurement of feed intake**
Feed intake was determined for individual birds for one week during the pre-treatment period in both groups and for the control group over the course of the treatment period (Fig. 7.1).

**Sample collection**
The birds were weighed and 20 ml samples of blood were collected at the start of the pre-treatment period. At the completion of the treatment period, 20 ml blood samples were collected and the birds were euthanased with Lethabarb as described in Chapter 2 (Fig. 7.1). The brains were removed from a subset of the animals (four control males, three starved males and one starved female), and the mediobasal hypothalamus and preoptic area tissues obtained and stored for RNA extraction and RPA analysis as described in Chapter 2.

**Carcass composition**
The carcass was weighed. The subcutaneous and visceral fat depots and the liver were removed and weighed to determine differences between the groups in adiposity for a subset of the animals used (same subset used for RPA analysis).

**RPA analysis**
RPA analysis was performed on the subset of samples collected. RNA was extracted as described in Chapter 2. The RPA was performed on total RNA extracted from the region containing the mediobasal hypothalamus and the preoptic area, using the RPA III kit (Ambion) as described in Chapter 2.

**Hormones and metabolites**
Radioimmunoassays were performed for corticosterone, insulin, triiodothyronine, thyroxine and glucagon for all samples as described in Chapter 2. β-hydroxybutyrate and urea assays were performed by the Agriculture Western Australia Animal Health Laboratory. β-hydroxybutyrate was measured using a spectrophotometric assay (McMurray *et al*, 1984). Urea was measured using the enzymatic UV test,
urease/glutamate dehydrogenase (GLDH) method. The urease/GLDH method incorporated assays for urea, using the Trace Urea (Urea Nitrogen) Reagent Kit (Roche), and GLDH, using the GLDH MPR 1 Reagent Kit (Roche). Both assays were performed on a Roche Cobas Mira S Automated Chemistry Unit. All samples for each metabolite were run in a single assay.

Data and statistical analysis
Before the starvation period commenced (pre-treatment) the birds were weighed and blood samples were collected and feed intake monitored, to enable within group comparisons to be made over time (Fig. 7.1). The expression of VIP and NPY were determined as values relative to the expression of β-actin for each sample. The carcass composition data for each tissue is expressed as a percentage of the live weight of the animal. The nature of differences between the starved and control groups was determined using Mann-Whitney U tests as described by Siegel (1956). The Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) was used to determine differences within groups over time using the Statview™ 512+ program (1986). Non-parametric statistical analysis was judged to be the most appropriate as two independent groups were sampled from, the sample size was small, and the number of birds in each group was not equal. The results are indicated as the mean ± s.e.m.
3. Results

RPA

There was no difference between control and starved male emus in the level of expression of either VIP or NPY after one week of starvation (p = 0.343 and p = 0.100 respectively) (Table 7.1).

**Table 7.1: The mRNA expression of NPY and VIP relative to β-actin of starved and control male emus.**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Control</th>
<th>Starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY/β-actin</td>
<td>1.07 ± 0.107</td>
<td>0.77 ± 0.168</td>
</tr>
<tr>
<td>VIP/β-actin</td>
<td>1.25 ± 0.284</td>
<td>1.82 ± 0.321</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m.

Feed intake

Feed intake did not differ between the groups for either sex in the pre-treatment period. There was no change in feed intake of the control males over time, however the feed intake of the females was higher during the treatment period (p = 0.0336) (Table 7.2).

**Table 7.2: The average daily feed intake of male and female emus in control and starved groups one week before, and during a subsequent one-week treatment period.**

<table>
<thead>
<tr>
<th></th>
<th>Control female</th>
<th>Starved female</th>
<th>Control male</th>
<th>Starved male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(kg/day)</td>
<td>(kg/day)</td>
<td>(kg/day)</td>
<td>(kg/day)</td>
</tr>
<tr>
<td></td>
<td>(N=4)</td>
<td>(N=5)</td>
<td>(N=4)</td>
<td>(N=3)</td>
</tr>
<tr>
<td>pre-treatment</td>
<td>0.45±0.07*</td>
<td>0.55±0.20</td>
<td>0.60±0.17</td>
<td>0.73±0.13</td>
</tr>
<tr>
<td>treatment</td>
<td>1.00±0.04*</td>
<td>-</td>
<td>0.85±0.22</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference within groups over time (p = 0.0336).
Live weight

Live weight did not differ between the groups at the start of the pre-treatment period or at the completion of the treatment period. There was no change in live weight of the control or starved males over time. However, live weight increased in the control and decreased in the starved females over time (p = 0.0336 and p = 0.0217 respectively) (Table 7.3).

<table>
<thead>
<tr>
<th></th>
<th>Control female (kg)</th>
<th>Starved female (kg)</th>
<th>Control male (kg)</th>
<th>Starved male (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N=4)</td>
<td>(N=5)</td>
<td>(N=4)</td>
<td>(N=3)</td>
</tr>
<tr>
<td>pre-treatment</td>
<td>51.0 ± 2.1*</td>
<td>52.4 ± 2.5a</td>
<td>49.5 ± 1.4</td>
<td>47.2 ± 3.1</td>
</tr>
<tr>
<td>treatment</td>
<td>53.6 ± 2.7*</td>
<td>50.9 ± 2.5a</td>
<td>49.3 ± 1.6</td>
<td>46.0 ± 3.3</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. The * and a indicate significant difference within groups over time (p < 0.05).

Carcass composition

The visceral, subcutaneous and total fat as a percentage of live weight did not differ between the groups. Liver weight as a percentage of live weight was higher in the control group at the completion of the treatment period (p = 0.029) (Table 7.4).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous adipose (%)</td>
<td>22.6 ± 1.09</td>
<td>22.8 ± 1.00</td>
</tr>
<tr>
<td>Visceral adipose (%)</td>
<td>9.90 ± 0.850</td>
<td>8.93 ± 0.869</td>
</tr>
<tr>
<td>Total adipose (%)</td>
<td>32.6 ± 0.33</td>
<td>31.8 ± 1.56</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>1.59 ± 0.092*</td>
<td>1.08 ± 0.150*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.029)
Chapter 7 - The effect of short-term starvation on the hypothalamic, expression of NPY and VIP during a period of elevated appetite

Hormones and metabolites

The insulin concentration was lower in the female starved group at the completion of the treatment period (p = 0.008), but not pre-treatment. The insulin concentration did not differ between the male groups at either time point. Within groups the insulin concentration decreased over time in the starved females (p = 0.022), but not the starved males or the control groups. There was a tendency for the insulin concentration to increase over time in the control female group (p = 0.072) (Table 7.5).

Table 7.5: Serum insulin concentrations of male and female emus in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female</th>
<th>Starved female</th>
<th>Control male</th>
<th>Starved male</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ng/ml)</td>
<td>(N=4)</td>
<td>(N=5)</td>
<td>(N=4)</td>
<td>(N=3)</td>
</tr>
<tr>
<td>pre-treatment</td>
<td>0.80 ± 0.04</td>
<td>0.82 ± 0.06*</td>
<td>1.06 ± 0.22</td>
<td>0.93 ± 0.07</td>
</tr>
<tr>
<td>treatment</td>
<td>1.45 ± 0.36*</td>
<td>0.70 ± 0.02*</td>
<td>1.18 ± 0.31</td>
<td>0.73 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.008). The * indicates significant difference over time (p = 0.0217).

The serum glucagon concentration was higher in the starved female group at the completion of the treatment period (p = 0.036). The serum glucagon concentration did not differ between the groups for the females pre-treatment, or the males pre-treatment or at the completion of treatment. Over time there was no difference in glucagon within either group for both sexes (Table 7.6).

Table 7.6: Serum glucagon concentrations of male and female emus in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female</th>
<th>Starved female</th>
<th>Control male</th>
<th>Starved male</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pg/ml)</td>
<td>(N=3)</td>
<td>(N=5)</td>
<td>(N=3)</td>
<td>(N=3)</td>
</tr>
<tr>
<td>pre-treatment</td>
<td>60.4 ± 18.8</td>
<td>75.2 ± 16.1</td>
<td>46.1 ± 18.6</td>
<td>118.8 ± 53.4</td>
</tr>
<tr>
<td>treatment</td>
<td>48.1 ± 26.8*</td>
<td>112.5 ± 15.9*</td>
<td>53.7 ± 29.8</td>
<td>139.1 ± 23.6</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.008).
Chapter 7 - The effect of short-term starvation on the hypothalamic, expression of NPY and VIP during a period of elevated appetite

The insulin/glucagon ratio was lower in both the male and female starved groups at the completion of treatment ($p = 0.050$ and $p = 0.036$ respectively). Over time there was a tendency for the ratio to decrease in the male and female starved groups, however this was not significant ($p = 0.055$ and $p = 0.069$ respectively). The control groups showed no change in their insulin/glucagon ratios over time (Table 7.7).

Table 7.7: The insulin/glucagon ratio of male and female emus in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female (N=3)</th>
<th>Starved female (N=5)</th>
<th>Control male (N=3)</th>
<th>Starved male (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-treatment</td>
<td>17.5 ± 4.8</td>
<td>13.6 ± 3.7</td>
<td>41.0 ± 19.9</td>
<td>13.7 ± 7.6</td>
</tr>
<tr>
<td>treatment</td>
<td>83.3 ± 52.0*</td>
<td>7.0 ± 1.4*</td>
<td>34.3 ± 10.9a</td>
<td>5.5 ± 0.8a</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between the female groups ($p < 0.05$). The a indicates significant difference between the male groups ($p < 0.05$).

The serum thyroxine concentration was higher in the starved females at the completion of treatment ($p = 0.032$). The concentration did not differ between the female groups at the commencement of the pre-treatment period. For the male groups the thyroxine concentration did not differ at the commencement of the pre-treatment period or the completion of treatment. Over time there was no difference in thyroxine within either group for both sexes (Table 7.8).

Table 7.8: Serum thyroxine concentrations of male and female emus in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female (nM/L, N=4)</th>
<th>Starved female (nM/L, N=5)</th>
<th>Control male (nM/L, N=4)</th>
<th>Starved male (nM/L, N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-treatment</td>
<td>2.59 ± 0.24</td>
<td>3.53 ± 0.32</td>
<td>3.61 ± 0.49</td>
<td>4.37 ± 0.29</td>
</tr>
<tr>
<td>treatment</td>
<td>2.88 ± 0.25*</td>
<td>4.17 ± 0.48*</td>
<td>2.73 ± 0.79</td>
<td>4.32 ± 0.40</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups ($p = 0.032$).
Chapter 7 - The effect of short-term starvation on the hypothalamic, expression of NPY and VIP during a period of elevated appetite

The serum triiodothyronine concentration was lower in the starved males than in the control males at the completion of treatment (p = 0.028). The triiodothyronine concentration did not differ between the female groups at either time-point or the male groups pre-treatment. Over time the triiodothyronine concentration increased in the male control group (p = 0.034). Over time the triiodothyronine concentration did not change for the starved males or either female group (Table 7.9).

Table 7.9: Serum triiodothyronine concentrations of male and female emus in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female (ng/mL)</th>
<th>Starved female (ng/mL)</th>
<th>Control male (ng/mL)</th>
<th>Starved male (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N=4)</td>
<td>(N=5)</td>
<td>(N=4)</td>
<td>(N=3)</td>
<td></td>
</tr>
<tr>
<td>pre-treatment</td>
<td>0.075 ± 0.018</td>
<td>0.136 ± 0.053</td>
<td>0.183 ± 0.036*</td>
<td>0.120 ± 0.061</td>
</tr>
<tr>
<td>treatment</td>
<td>0.090 ± 0.033</td>
<td>0.092 ± 0.019</td>
<td>0.223 ± 0.029**a</td>
<td>0.067 ± 0.015*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.028). The " indicates significant difference over time (p = 0.034).

The triiodothyronine/thyroxine (T3/T4) ratio was lower in the starved than in the control males at the completion of the treatment (p = 0.028). The T3/T4 ratio did not differ between the female groups at either time-point, or the male groups pre-treatment. Within groups there was no difference in the T3/T4 ratio for either sex over time (Table 7.10).

Table 7.10: Serum T3/T4 ratio (1 x 10^-5) of male and female emus in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female (N=4)</th>
<th>Starved female (N=5)</th>
<th>Control male (N=4)</th>
<th>Starved male (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-treatment</td>
<td>7.26 ± 2.22</td>
<td>3.59 ± 1.79</td>
<td>3.76 ± 0.81</td>
<td>5.52 ± 2.39</td>
</tr>
<tr>
<td>treatment</td>
<td>15.53 ± 6.01</td>
<td>2.09 ± 0.59</td>
<td>4.37 ± 1.77*</td>
<td>2.96 ± 0.62*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.028).
Chapter 7 - The effect of short-term starvation on the hypothalamic, expression of NPY and VIP during a period of elevated appetite

The serum corticosterone concentration did not differ between the groups for either sex at the commencement of the pre-treatment period or at the completion of treatment. Over time there was no change in the concentration of corticosterone within any group (Table 7.11).

Table 7.11: Serum corticosterone concentrations of male and female emus in each group in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female (ng/mL)</th>
<th>Starved female (ng/mL)</th>
<th>Control male (ng/mL)</th>
<th>Starved male (ng/mL)</th>
</tr>
</thead>
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<tr>
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<td>(N=4)</td>
<td>(N=5)</td>
<td>(N=4)</td>
<td>(N=3)</td>
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<tr>
<td>pre-treatment</td>
<td>1.26 ± 0.14</td>
<td>1.84 ± 0.88</td>
<td>0.82 ± 0.12</td>
<td>0.78 ± 0.19</td>
</tr>
<tr>
<td>treatment</td>
<td>2.60 ± 0.86</td>
<td>1.41 ± 0.15</td>
<td>1.47 ± 0.19</td>
<td>1.46 ± 0.46</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m.

The serum β-hydroxybutyrate concentration did not differ between the groups for either sex at the commencement of the pre-treatment period. At the completion of treatment the β-hydroxybutyrate concentration was higher in the starved males than the control males, but did not differ between groups for the females. Over time there was no change in the β-hydroxybutyrate concentration within any group, however there was a trend for increased β-hydroxybutyrate in the starved males (p = 0.055) (Table 7.12).

Table 7.12: Serum β-hydroxybutyrate concentrations of male and female emus in each group in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female (mmol/L)</th>
<th>Starved female (mmol/L)</th>
<th>Control male (mmol/L)</th>
<th>Starved male (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N=4)</td>
<td>(N=5)</td>
<td>(N=4)</td>
<td>(N=3)</td>
</tr>
<tr>
<td>pre-treatment</td>
<td>0.62 ± 0.26</td>
<td>0.34 ± 0.17</td>
<td>0.05 ± 0.03</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>treatment</td>
<td>0.32 ± 0.31</td>
<td>0.83 ± 0.40</td>
<td>0.29 ± 0.23*</td>
<td>1.41 ± 0.15*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.055)
The serum urea concentration did not differ between the groups for either sex pretreatment or for the females at the completion of treatment. At the completion of treatment the urea concentration was lower in the starved males than the control males ($p = 0.022$). Over time the urea concentration decreased within the starved female group and exhibited a decreasing trend in the starved males ($p = 0.022$ and $p = 0.055$ respectively). Within the control male and females groups there was no change over time in the concentration of urea (Table 7.13).

**Table 7.13:** Serum urea concentrations of male and female emus in each group in each group before and after a 7 day treatment period.

<table>
<thead>
<tr>
<th></th>
<th>Control female (mmol/L)</th>
<th>Starved female (mmol/L)</th>
<th>Control male (mmol/L)</th>
<th>Starved male (mmol/L)</th>
</tr>
</thead>
<tbody>
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<td>(N=4)</td>
<td>(N=5)</td>
<td>(N=4)</td>
<td>(N=3)</td>
</tr>
<tr>
<td>pre-treatment</td>
<td>1.70 ± 0.30</td>
<td>1.38 ± 0.27*</td>
<td>1.63 ± 0.34</td>
<td>2.03 ± 0.19</td>
</tr>
<tr>
<td>treatment</td>
<td>1.75 ± 0.22</td>
<td>0.70 ± 0.13*</td>
<td>1.78 ± 0.26*</td>
<td>0.87 ± 0.19*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups ($p = 0.028$). The a indicates significant difference over time ($p = 0.022$).
4. Discussion

The hypothesis that NPY mRNA expression would be elevated by short-term starvation in male emus was not supported by the results. However, the hypothesis that VIP mRNA expression would be unchanged was supported by the results. The hypothesis that short-term starvation induces metabolic changes was partially supported by the results with one week of starvation decreasing insulin concentrations in the female starved group below that of both their pre-treatment concentration and the completion of treatment concentration of the controls. In the females the glucagon concentration was also higher at the completion of treatment in the starved group. This indicates that females may be more sensitive to the effects of starvation than males. Effects on the thyroid hormones varied between the sexes. The thyroxine concentration was higher in starved females at the completion of treatment, while the triiodothyronine concentration and the T3/T4 ratio were lower in starved males at the completion of treatment. This may again indicate a greater sensitivity to starvation in the female emu as the differences in the male values can largely be explained by an increase in the triiodothyronine concentration of the controls at the completion of the treatment period. Given the anorexia male emus exhibit when incubating eggs, and the fact that females during the breeding season only exhibit a reduced appetite, a greater resistance to starvation in male emus is a reasonable finding. The corticosterone concentration did not increase in response to starvation in either sex, indicating that seven days of starvation does not impose a large stress, as measured by corticosterone concentration, on the emu.

The concentrations of metabolites were changed by starvation, and as with the hormone concentrations these changes differed between the sexes. The concentration of β-hydroxybutyrate was higher in the starved male group at the completion of treatment. Rather than indicating greater sensitivity to starvation, this may indicate an adaptation in males that enables them to switch more quickly to the utilisation of adipose stores in response to starvation than females. The starved female group showed a non-significant increase in the concentration of β-hydroxybutyrate, and along with the initiation of metabolic changes to conserve energy this may indicate that they employ a different strategy to males in response to starvation. The concentration of urea decreased over time in the female starved group and was lower at the completion of treatment in the male starved group. This result was unexpected but can be explained in terms of the diet and age of these animals. The animals used in this experiment were mature and received a
commercial diet containing 16% protein. The reduction in urea concentration indicates that the protein content of this diet exceeds the mature emus requirements. In the control group the higher urea concentration is due to the need to breakdown and excrete excess dietary protein.

The absence of changes in NPY mRNA expression in the mediobasal hypothalamus/preoptic area or corticosterone concentration in response to starvation, and the variable effects of starvation on metabolism implies two things. First, that the emu is more resistant to starvation than other species, and any effects of starvation on NPY expression or the concentration of corticosterone may only be observed after prolonged periods. One week of starvation did not decrease either the subcutaneous or visceral adipose reserves, although it did significantly decrease the liver weight as a proportion of live-weight. The decreasing liver weight (more than 30% lower) represents the utilisation of stored glycogen reserves. A suitable period to examine the role of NPY in the control of appetite in the emu would have been such that the circulating concentration of corticosterone was increased, the stored glycogen reserves were depleted by more than 30% and catabolism of stored triglycerides from adipose tissue was initiated. Second, that NPY does not contribute significantly to the control of appetite in emus. Research in mice has shown that multiple pathways are involved in the regulation of appetite and body weight, and in the absence of NPY, compensate to maintain adipose reserves in the correct range (Erickson et al, 1996). It is therefore plausible that in the emu one of these other pathways is of greater importance to the control of appetite and bodyweight than NPY. Central pathways that could be of importance to the control of appetite in the emu include those involving cocaine and amphetamine-regulated transcript (CART), orexin/hypocretin, corticotrophin-releasing hormone (CRH), proopiomelanocortin (POMC) and agouti-related protein (AGRP).

In conclusion, subjecting emus to one week of starvation does not increase NPY gene expression or induce the range of hormonal changes that are associated with starvation in other species. This may be due to a greater tolerance to starvation in emus. Given that male emus exhibit anorexia for a period of eight weeks during incubation, it is not surprising that a single week of starvation failed to induce the classical starvation responses. The absence of any change in NPY mRNA expression in response to short-term starvation could also be due to a more limited role of NPY in appetite control than is
observed in other species. This experiment did not clarify the roles of NPY and VIP in appetite control or the possibility of mediation of NPY’s effects on appetite by corticosterone. Performing this experiment over a longer time period may have yielded more information on the control of appetite in the emu, as would comparisons between the expression of NPY during the breeding season (minimal appetite) and the summer months (maximal appetite).
General Discussion
The emu has great potential as a commercial livestock species. The major drawback to achieving this potential is the seasonal decrease in appetite and adiposity that they exhibit in response to decreasing photoperiod. Decreased appetite, and particularly the ensuing decreased adiposity, are a problem because fat is the most profitable component of the carcass. In a commercial production scenario the decreased appetite and adiposity restricts the viable window for slaughter to a brief period during the summer months. As a consequence of this restriction the availability of emu products (i.e. meat, leather and fat) are limited and market development is hampered. The aim of this thesis has been to improve the understanding of how appetite and adiposity are controlled in the emu. Improving our understanding of these controls is essential for identifying mechanisms to manipulate appetite and adiposity, and for developing techniques to assess the susceptibility of the emu to these manipulations.

Emus might have different mechanisms to control appetite and adiposity than those described in other species. During the course of this thesis emus have demonstrated a striking resistance to the manipulation of both appetite and adiposity (see Chapters 4 and 5) and tolerance to short-term starvation (see Chapter 7). Emus are also well adapted to survive prolonged negative energy balance during the anorexia of incubation (see Chapter 6). Emus have evolved in a harsh environment in which food is often scarce (Davies, 1978). As such, the unique features of the emu demonstrated during the course of this thesis may result from adaptations to this harsh environment. To cope with food scarcity and the demands of reproduction the emu has evolved efficient mechanisms to maintain energy reserves at an appropriate level. These mechanisms include strategies for coping with starvation that diminish the stress response and appetite stimulation, and thereby minimise discomfort and the negative effects of continuously elevated stress hormones on the animal (see Chapter 7). The efficient and rigid control of appetite and adiposity allows the emu to resist treatments that are known to alter appetite and adiposity in other species, such as, dexamethasone treatment (see Chapter 5) and the consumption of high fat diets (see Chapter 4). As such, it may not be possible to maintain high appetite and adiposity in the emu during the breeding season.

My work has focused on how to prevent appetite and adiposity decreasing. It is possible that this is the wrong approach for tackling the problem of a limited slaughter window for producers. I assumed that appetite was decreasing during the reproductive period and that
the emus could not meet their requirements during this period when compared to the large amount of food ingested over summer. In fact, the increase in appetite during summer may be part of a strategy of over feeding to increase energy reserves and support the demands of reproduction during winter. Premigratory fattening is an analogous situation where appetite is increased to increase adipose reserves and enable migrating species to cover large distances when a food supply is unreliable or unavailable (Stevens, 1996). For a species to survive it must reproduce (Jones, 1999). Therefore factors that influence the success of reproduction, such as the control of energy reserves, come under intense selection pressure. Adaptations that have evolved under such intense selection are unlikely to be overcome with ease. Therefore, it may be better to investigate the mechanism controlling the stimulation of appetite at the completion of incubation. Studies into the rapid and large increase in food intake of the male emu observed at the cessation of incubation would be valuable and could provide feasible mechanisms for increasing appetite earlier and thereby extending the slaughter window.

Natural selection has encouraged the evolution of photoperiod-related controls of appetite and adiposity in the emu that strongly resist interference (Blache & Martin, 1999; Blache et al, 2001b). The photoperiodic control of appetite most likely involves a complicated network involving interactions between hypothalamic neuropeptides, peripheral metabolic and reproductive hormones, and photoperiod-related factors. The photoperiodic control of appetite during the reproductive period will be difficult to characterise fully and may be impossible to override. A promising approach to manipulate appetite and adiposity during the reproductive period would be to alter the perception of photoperiodic changes by the emu. In mammals, melatonin is a key component of photoperiodicity and its administration can inhibit reproduction (Edmonds & Stetson, 1994; Lincoln, 1994; Horton & Yellon, 2001). However, in birds, it appears that melatonin may not be involved in the control of reproduction by photoperiod, although, the role of melatonin in emus has never been investigated (Meyer, 1998). Other factors that could provide insights into the photoperiodic control of appetite and potential mechanisms for disrupting it include; norepinephrine, gonadotrophin-releasing hormone, and proopiomelanocortin (POMC) and agouti-related protein (AGRP) (Kriegsfeld et al, 2000; Mercer et al, 2000a & 2000b; Imundo et al, 2001), further investigations of these pathways could lead to ways to manipulate the effect of photoperiod on appetite in emus.
In conclusion, I have explored several avenues to prevent the loss of appetite and adiposity during the breeding season. Methods that are known to increase appetite and adiposity in other species have little or no effect in emus. It is possible that investigations into the mechanisms that trigger the rapid and large increase in appetite and adiposity after the breeding season could yield new approaches for manipulating appetite and adiposity in the emu to extend the slaughter window. Another area for investigation that could provide methods for manipulating appetite and adiposity is the neural basis for the control of appetite by photoperiod.
Bibliography

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<tr>
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<tr>
<td>1.</td>
<td>Websites</td>
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<tr>
<td>2.</td>
<td>Computer programs</td>
</tr>
<tr>
<td>3.</td>
<td>Articles and books</td>
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1. Websites
Bureau of Meterology
National Centre for Biotechnology Information
Rural Industries Research and Development Corporation
NIH Image program, U.S. National Institutes of Health
http://rsb.info.nih.gov/nih-image/

2. Computer programs

3. Articles and books
Adams BM (1968) Effect of cortisol on growth and uric acid excretion in the chick,  
Adams NR & Sanders MR (1992) Improved feed intake and body weight change in sheep  
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151:110-125.
Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, Crow TJ,  
Tatemoto K & Polak JM (1983) Neuropeptide Y distribution in human brain,  
Ahrén B & Scheurink AJW (1998) Marked hyperleptinemia after high-fat diet associated  
with severe glucose intolerance in mice, European Journal of Endocrinology.  
139:461-467.
Ahrén B (1999) Plasma leptin and insulin in C57BL/6J mice on a high-fat diet: relation to  


Harvey S, Scanes CG, Chadwick A & Bolton NJ (1978) Influence of fasting, glucose and insulin on the levels of growth hormone and prolactin in the plasma of the domestic fowl (Gallus domesticus), Journal of Endocrinology. 76:501-506.


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Mateos GG, Sell JL & Eastwood JA (1982) Rate of food passage (transit time) as influenced by level of supplemental fat, Poultry Science. 61:94-100.


Mawson P (1992) The emu, APB Infonote 21/92. in: Emu farming, Department of Agriculture Western Australia miscellaneous publication.


Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA & Mobbs CV (1997) Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting in ob/ob and db/db mice, but is stimulated by leptin, *Diabetes.* 47:294-297.


Smetana P (1994) *Emu farming*, Department of Agriculture Western Australia miscellaneous publication.


Bibliography


### Abbreviations used in the thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic</td>
</tr>
<tr>
<td>AGRP</td>
<td>Agouti-related protein</td>
</tr>
<tr>
<td>amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ANSA</td>
<td>8-anilino-1-naphthalene sulphonic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B₀</td>
<td>Zero binding</td>
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<td>Body mass index</td>
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<td>Bovine serum albumin</td>
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<td>CART</td>
<td>Cocaine and amphetamine-related transcript</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CER</td>
<td>Cerebellum</td>
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<td>CH₃COONa</td>
<td>Sodium acetate</td>
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<tr>
<td>C₈H₁₁N₂NaO₃</td>
<td>Barbital sodium</td>
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<tr>
<td>CP</td>
<td>Choroid plexus</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
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<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
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<td>CSIRO</td>
<td>Commonwealth Scientific and Industrial Research Organisation</td>
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<td>CTP</td>
<td>Cytocine triphosphate</td>
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<td>Diabetes genotype</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>GPB</td>
<td>Gelatin phosphate buffer</td>
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<tr>
<td>GTP</td>
<td>Guanidine triphosphate</td>
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<td>IU</td>
<td>International units</td>
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<td>KI</td>
<td>Potassium iodide</td>
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<td>Lithium chloride</td>
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<td>MBH</td>
<td>Mediobasal hypothalamus</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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### Appendix 1 - Abbreviations

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<td>NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Sodium azide</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Disodium hydrogen orthophosphate</td>
</tr>
<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;.2H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Sodium dihydrogen orthophosphate</td>
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<td>Sodium metabisulphite</td>
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<td>NGPS</td>
<td>Normal guinea pig serum</td>
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<td>NIH</td>
<td>National Institute of Health</td>
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<td>Obese genotype</td>
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<td>PIPES</td>
<td>Piperazine-N,N’-bis[2-ethane sulfonic acid]</td>
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<td>POA</td>
<td>Preoptic area</td>
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<td>POMC</td>
<td>Proopiomelanocortin</td>
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<td>PS</td>
<td>Pituitary stalk</td>
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<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
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<td>Radioimmunoassay</td>
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<td>RIRDC</td>
<td>Rural Industries Research and Development Corporation</td>
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<td>Revolutions per minute</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
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<td>SSC</td>
<td>Sodium chloride sodium citrate</td>
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<td>Taq</td>
<td>Taq polymerase enzyme</td>
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<tr>
<td>TC</td>
<td>Total counts</td>
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<tr>
<td>TEMED</td>
<td>N,N,N’,N’-tetra-methyl-ethylenediamine</td>
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<td>TRH</td>
<td>Thyroid releasing hormone</td>
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<td>UTP</td>
<td>Uracil triphosphate</td>
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<tr>
<td>UWA</td>
<td>University of Western Australia</td>
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<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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<td>VLDL</td>
<td>Very low-density lipoprotein</td>
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<tr>
<td>VMH</td>
<td>Ventromedial hypothalamic nucleus</td>
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Appendix 2 – List of suppliers and feed composition

Alphabetical list of suppliers

Consumables, chemicals and equipment

Ambion, Inc.
2130 Woodward
Austin
TX 78774-1832
USA
http://www.ambion.com/
Distributed in Australia by GeneWorks Pty Ltd

Amersham Biosciences Pty Ltd
Unit 1, number 22 Hudson Avenue
Castle Hill
NSW 2154
AUSTRALIA

Beckman
(now Beckman Coulter Australia Pty Ltd)
Unit C, 24 College Street
Gladesville
NSW 2111
AUSTRALIA
http://www.beckman.com/

Biogenesis Immuno Diagnostics
(now Biogenesis Ltd)
Technology Road
Poole
BH17 7DA
UK
http://www.biogenesis.co.uk/

BioRad Laboratories Pty Ltd
Unit 1, Block Y
Regents Park Industrial Estate
391 Park Road
Regents Park
NSW 2143
AUSTRALIA
http://www.bio-rad.com/

Boehringer Mannheim
(acquired by Roche Products Pty Ltd)
4-10 Inman Road
Dee Why
NSW 2099
AUSTRALIA
http://www.roche.com/

Clontech
(now BD Biosciences Clontech)
4 Research Park Drive
Macquarie University
Research Park
North Ryde
NSW 2113
AUSTRALIA
http://www.clontech.com/

Endocrine Sciences Products
(now Endocrine Sciences RIA Reagents)
4301 Lost Hills Rd
Calabasas
CA 91301
USA

Genesearch Pty Ltd
14 Technology Drive
Arundel
QLD 4214
AUSTRALIA

GeneWorks Pty Ltd
39 Winwood Street
Thebarton
SA 5031
AUSTRALIA
GIBCO  
(now Invitrogen Life Technologies)  
In Israel distributed by  
RHENIUM Ltd  
PO Box 3580  
91035 Jerusalem  
ISRAEL  
http://www2.lifetech.com/  

Hybaid  
(now Thermo Hybaid)  
Thermo Biosciences GmbH  
Sedanstr. 18  
D-89077 Ulm  
GERMANY  
http://www.hybaid-gmbh.com/  

Ilium Veterinary Products  
(supplied by Troy Laboratories Pty Ltd)  

Integrated Sciences  
PO Box 196  
East Kew  
Melbourne  
VIC 3102  
AUSTRALIA  

Linco Research, Inc.  
14 Research Drive  
St Charles  
Missouri 63304  
USA  

Medisense  
(now produced by Abbott Laboratories)  
Abbott Diagnostics Division  
PO Box 394  
North Ryde  
NSW 2113  
AUSTRALIA  
http://www.medisense.com/  

NEN Life Science  
549 Albany Street  
Boston  
MA 02118-2512  
USA  

New England Biolabs  
http://www.neb.com/  
Distributed in Australia by Genesearch Pty Ltd  

Packard  
(now PerkinElmer Life Sciences)  
PerkinElmer Pty Ltd  
16-18 Kingsley Close  
Rowville  
Melbourne  
VIC 3178  
AUSTRALIA  

Pharmacia Biotech  
(now Amersham Biosciences Pty Ltd)  

Promega Corp. Australia  
37 Nelson Street  
Annandale  
NSW 2038  
AUSTRALIA  
http://www.promega.com/  

QIAGEN Pty Ltd  
PO Box 25  
Clifton Hill  
VIC 3068  
AUSTRALIA  
http://www.qiagen.com/  

Sarstedt Australia Pty Ltd  
PO Box 90  
Ingle Farm  
SA 5098  
AUSTRALIA  
http://www.sarstedt.com/
Appendix 2 – List of suppliers and feed composition

Sigma
(now Sigma-Aldrich Pty Ltd)
Sydney
AUSTRALIA
http://www.sigma.aldrich.com/

Stratagene
11011 N. Torrey Pines Road
La Jolla
CA 92037
USA
http://www.stratagene.com/
Distributed in Australia by Integrated
Sciences

Tel-test Inc.
PO Box 1421
Friendswood
TX 77546
USA
http://www.isotexdiagnostics.com/
Distributed in Australia by GeneWorks Pty
Ltd

Turner Design Inc
845 W. Maude Avenue
Sunnyvale
CA 94085
USA
http://www.turnerdesigns.com/

Virbac
15 Pritchard Place
Peakhurst
NSW 2210
AUSTRALIA

Whatman International Ltd
Whatman House
St Leonards Road
20/20 Maidstone
Kent
ME16 0LS
UK
http://www.whatman.com/

Diet components and commercial feeds

Bayer
PO Box 1045
3 De Laeter Way
Bentley
WA
AUSTRALIA

Education Colours Pty Ltd
PO Box 657
Croydon
VIC 3136
AUSTRALIA

Davison Industries
Pinjarra
WA
AUSTRALIA

Glen Forrest Stockfeeders
3150 Great Eastern Highway
Glen Forrest
WA 6076
AUSTRALIA
Feed composition

Glen Forrest Stockfeeders emu breeder pellet:

- 15% protein
- 7.6% crude fibre
- 3.7% fat

providing 10.5MJ/kg of metabolisable energy.
## Appendix 3

Response to comments made by thesis examiners

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Chapter 2.

Question 1. Were feed refusals sub-sampled and analysed to determine actual intake i.e. was there any scope for birds to select differently from diet specifications?
Refusal margins were not calculated as the amount fed to each bird was adjusted continuously in response to changes in intake. Refusals were not sub-sampled as the diet was pelleted and the components had been finely ground making differential selection less likely. The two diets created for experiment 1 (Chapter 4) were also sifted at each weighing and refilling to remove any powder that had accumulated, further reducing the opportunity for differential selection of the diet components.

Question 2. Corticosterone assay – why were different sized tubes used for samples and standards?
As the standards were not extracted they were set up in a tube appropriate to the volume used for the assay. The extraction of corticosterone was performed in larger tubes that were appropriate for the volume of iso-octane/hexane used the resulting organic layer was then transferred to the smaller tubes for drying down and continuation of the assay.

Question 3. Glucagon assay – what is aprotinin? why was normal guinea pig serum (NGPS) used?
Aprotinin is added to the assay to protect against protease activity. NGPS was added to the assay to aid precipitation.

Question 4. Insulin assay – why was 0.1% gelatine added to the assay buffer?
Insulin may be present in BSA preparations, therefore gelatine was added in preference to BSA to prevent confounding of the results by insulin contamination. Additionally the use of gelatine in the assay buffer was recommended in the original assay method.

Question 5. LH assay – why were fractions eluted into buffer plus BSA?
The BSA stops the radioactively labelled LH from sticking to the tube.
Question 6. T3/T4 assays – why was stripped plasma added to the standards in both assays? why were glass tubes used?

Stripped plasma was added to the tubes to provide a blank effect for plasma in the assay. Glass tubes were used because the assay was performed in a small volume and with glass tubes droplets don’t stick to the sides and therefore give less error.

Question 7. Why wasn’t glucagon assay 2 with 66% binding repeated?

The components used in the glucagon assay were mainly purchased from a commercial supplier and the cost of repeating this assay was prohibitive.
Chapter 4.

Question 1. Figure 1.1 suggests through spring and summer that males gain significant live weight, why then did neither group gain weight during the experiment?

The birds used in this experiment were caught fortnightly for weighing and bleeding. The handling involved in this process, in particular the weighing procedure, can be stressful for the bird. This stress may have had a negative effect on appetite in these birds. Indeed, a decrease in food intake was observed in all birds after handling. Their food intake slowly recovered to pre-handling levels over two weeks, at which time they were handled again. In subsequent experiments weighing of birds was kept to a minimum to try and avoid this effect.

Question 2. As neither group gained weight changes in total fat would have to occur through a decrease in the weight of another tissue, is this suggested anywhere in the literature? Or were you never going to get a response because weight did not change?

The literature does not indicate that adipose tissue weight can increase when weight is constant. However, there is evidence that the type of fat consumed can influence the oxidation rate of fat in chickens and therefore the partitioning of energy into lean or fat tissue (Crespo & Esteve-Garcia, 2002; Newman et al, 2002). Saturated fats induce greater abdominal adipose deposition than unsaturated fats in chickens (Vilà & Esteve-Garcia, 1996; Sanz et al, 1999 & 2000). As such, a diet high in saturated fat could cause adiposity to increase despite only moderate changes in weight. In combination though the absence of increases in weight gain in this experiment and the use of unsaturated fat in the diet did make it unlikely that an increase in adiposity would be observed.

Question 3. What are the hormonal impacts on synthesis, degradation and accretion rates in fat and muscle?

The key hormones involved in the storage and mobilisation of energy reserves are insulin, glucagon and glucocorticoids. They affect synthesis, degradation and accretion rates of fat and muscle through various mechanisms.

1) Insulin:
- in the liver increases glucose uptake, inhibits gluconeogenesis, and increases lipogenesis and the release of VLDL into the peripheral circulation (Stevens, 1996),
- in adipose tissue, increases glucose uptake, inhibits free fatty acid release, stimulates
the conversion of glucose to fat and stimulates lipoprotein lipase concentrations (Griminger, 1986; Hazelwood, 1986), sparing the triglyceride stores and increasing adipose deposition,
- in muscle increases the rate of glucose uptake, facilitates the formation of glycogen from glucose, inhibits glycogen mobilisation and stimulates the uptake of amino acids (Bentley, 1998).

2) Glucagon:
- in the liver mobilises energy reserves by inhibiting lipogenesis and stimulating gluconeogenesis (Stevens, 1996),
- in adipose tissue stimulates lipolysis (Stevens, 1996), releasing free fatty acids into the peripheral circulation (Braganza et al, 1973),
- in muscle tissue mobilises glycogen and amino acids (Bentley, 1998).

3) Glucocorticoids:
- in the liver stimulate lipogenesis and gluconeogenesis (Griminger, 1986; Stevens, 1996),
- in adipose tissue stimulate lipolysis (Griminger, 1986; Stevens, 1996),
- in muscle mobilise protein and deaminate amino acids that are then used as a substrate for gluconeogenesis in the liver (Bentley, 1998).

**Question 4. Does glucagon have lipolytic potential in emus as in other avian species?**

Glucagon was elevated in the high fat group, therefore was intake depressed because of the high fat diet and glucagon increasing lipolysis and therefore the circulating energy levels were very high and intake was suppressed?

It has not been assessed in the emu but it is likely that glucagon does have the same lipolytic potential observed in other avian species. The results for glucagon indicate a trend for elevated glucagon in the high fat group at a single time point. This is at the end of the diet changeover period when the birds showed the largest reductions in food intake. As such, the raised glucagon levels are more likely to be a consequence of decreased food intake, rather than a cause. It is possible that the decreased feed intake led to an increase in glucagon to stimulate lipolysis and meet the energetic requirements of the animals.
Chapter 5.

**Question 1.** How do terminal fat scores relate to actual measurements of carcass adiposity? Furthermore a correlation analysis would help to calibrate the subjective scoring system for future use.

The fat scoring system used has been previously shown to correlate well with adiposity of emu carcasses as assessed by ultrasound (Mincham *et al.*, 1998). The results for fat scoring performed during experiment 2 (Chapter 5) exhibit a strong positive relationship between fat score and the percentage total adiposity (correlation coefficient = 0.918) (Figure A3.1). Analysis of variance indicates that this relationship is significant (F ratio = 53.48, => p value < 0.001).

![Figure A3.1: Correlation analysis of the relationship between fat score and percentage total adiposity of female emus in experiment 2 (saline treated = open circles and dexamethasone treated = solid circles).](image-url)
Question 2. How representative are single LH determinations? What are the LH secretory dynamics in female emus? Why not measure ovarian steroids? And/or FSH?

In chickens, adult cockerels but not laying hens have been shown to have episodic LH release (Wilson & Sharp, 1975). In turkeys LH secretion by females differs between photosensitive and photostimulated birds. Photosensitive birds have LH pulses of low frequency and high amplitude with low baseline LH concentrations and photostimulated birds have a high baseline LH concentration with few pulses of low amplitude and short duration (Chapman et al, 1994).

In emus serial blood sampling did not show any evidence of LH pulsatility in the female emu (Van Cleeff, 2001). As such, single determinations can be considered to accurately represent LH concentration in the emu. Ovarian steroids and FSH were not measured due to the cost and time that would have been required to develop and validate the assays for emus.

Question 3. Is there a reference for reduced feed intake leading to reduced ovarian weight in emus/birds (over time course here of 3 weeks with appetite reduced by 30-70%)?

Feeding chickens a diet high in zinc or fasting them for 10 days decreases food intake and ovary weight (McCormick & Cunningham, 1987). Restricted feeding of broilers to 45% of ad libitum also results in lower ovary weights (Waddington & Hocking, 1993). However, glucocorticoid administration has been shown to decrease ovary weight in the hen in the absence of changes in live weight or food intake (Williams et al, 1985). As such, the suppression of ovary development observed in experiment 2 (Chapter 5) may be due initially to treatment with dexamethasone and compounded by the reduction in food intake observed at the end of the experiment.

Question 4. Lipid infiltration of the emu liver in response to dexamethasone treatment – discuss/reference more fully.

Dexamethasone treatment can cause lipid infiltration of the liver (Franco-Colin et al, 2000; Letteron et al, 1997). Dexamethasone causes lipid infiltration in several ways:

1) increasing the activity of phosphatidate phosphohydrolase and thereby the synthesis of neutral lipids from glycerol phosphate (Pittner et al, 1995),

2) inhibiting medium- and short-chain acyl CoA dehydrogenation and hepatic lipid secretion (Letteron et al, 1997)
1) inducing haptoglobin production, an acute phase protein associated with hepatic lipodosis (Higuchi et al, 1994).

The increased liver weight observed following chronic treatment with dexamethasone during experiment 2 (Table 5.2) and the associated difference in appearance of the liver between the two groups (Figure A3.2) are consistent with lipid infiltration.

![Liver tissue from female emus following chronic treatment with either saline (left) or dexamethasone (right).](image)

**Figure A3.2:** Liver tissue from female emus following chronic treatment with either saline (left) or dexamethasone (right).

**Question 5. Increased blood glucose result is not discussed. Treatment reduced the insulin:glucose ratio? Why did this not effect NPY?**

The increased blood glucose resulted from the action of dexamethasone to inhibit peripheral oxidation and utilisation of glucose. The elevation of blood glucose, in the absence of changes in the concentration of insulin, did result in a decrease in the insulin:glucose ratio. Elevated insulin concentrations act to decrease NPY gene expression (Schwartz et al, 1991 & 1992). Therefore it is unlikely that insulin was influencing NPY expression in this experiment. In the arcuate nucleus glucose-sensitive neurons overlap with NPY-containing neurons (Muroya et al, 1999). A decrease in central glucose concentration activates these neurons causing them to release NPY and thereby stimulate feeding and normalisation of peripheral and central glucose concentrations. As peripheral glucose concentrations were increased in experiment 2, it is unlikely that they would have stimulated NPY by this mechanism. Acute stimulation of blood glucose concentration is associated with elevations of NPY gene expression and corticosterone concentration (Wang et al, 1999). As corticosterone was depressed by dexamethasone
treatment in experiment 2, it is possible that the absence of an effect of glucose on NPY expression reflects a requirement for corticosterone to potentiate any effects of elevated glucose on NPY gene expression.

**Question 6. Why did animals fed ad libitum not gain weight?**

This experiment involved weekly handling of the animals for injections and bleeding, and as for experiment 1 this regular handling was most likely a significant stressor for the birds that may have reduced their food intake. The pattern of reduced feed intake after handling observed in experiment 1 was not seen during this experiment. However, the greater frequency of handling may have prevented appetite recovering in the interim period.
Chapter 6.

Question 1. Compare food intake of incubating and non-incubating birds to "control" birds during the summer.

The food intake of non-incubating emus during autumn (short daylength) and summer (long daylength) on-farm, and under equivalent photoperiod treatments, was higher in both experiments in the summer/long day groups (Blache & Martin, 1999; O'Malley, 1996).

Question 2. Does blood sampling during incubation constitute a major disturbance? Do emus abstain while sitting but get up at certain times to feed?

Blood sampling does not constitute a major disturbance during incubation. Birds remained on the nest during the procedure and generally showed no response to the handling involved. The birds abstain from eating while sitting and very rarely leave the nest. During the incubation period they exhibit anorexia.

Question 3. Also the time of day of killing might be important? Could there be diurnal changes in NPY expression controlling the moment-to-moment drive to eat?

The birds were always killed in the morning. As such, diurnal changes in NPY expression should not have confounded the results of this experiment. Diurnal changes in NPY expression could control the moment-to-moment drive to eat in emus. Investigation of the eating patterns of emus has revealed that they only eat during the light period and that the difference in food intake observed between birds on LD and SD is due to differences in the time spent eating rather than in the frequency of meals (Blache & Martin, 1999). On LD, but not SD, the final 4 hours of light was associated with a two-fold decrease in feeding frequency. In rats, NPY gene expression in the hypothalamus increases during the light phase, decreases just prior to the onset of the dark phase and remains low throughout the dark phase (Xu et al, 1999). Food intake in rats is highest during the dark phase and lowest during the light phase (Lu et al, 2002). Therefore, increased NPY expression during the light phase may be a response to negative energy balance during this period that ensures appetite is high when the dark phase begins. The decreased NPY expression during the period of maximal appetite can be explained by increases in peripheral signals such as insulin and glucose that result from eating. If NPY gene expression shows diurnal changes that regulate appetite in a similar
manner to rats it could be expected that NPY gene expression would be highest during the
early hours of the morning and maintained at a lower constant level throughout the day.

**Question 4. How was it possible to quantify lanes 1 and 2?**

It is possible that these lanes are saturated, the data from the image analysis program did
however appear reasonable and the data was therefore retained to keep the birds in the
incubating group at a reasonable number. Leaving the data for these lanes out of the Mann-
Whitney U test does not change the interpretation of the results (Table A3.1).

Table A3.1: The mRNA expression of NPY and VIP relative to β-actin of incubating (n=3) and non-incubating
(n=6) male emus.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Incubators</th>
<th>Non-incubators</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY/β-actin</td>
<td>0.68 ± 0.075</td>
<td>0.53 ± 0.083</td>
</tr>
<tr>
<td>VIP/β-actin</td>
<td>0.66 ± 0.030*</td>
<td>0.47 ± 0.055*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m. An * indicates significant difference between groups (p = 0.019).

**Question 5. The trend to increased NPY could relate to the increased corticosterone, prolactin or reduced insulin:glucagon ratios?**

Using correlation analysis to investigate relationships between NPY gene expression and
either corticosterone, prolactin or the insulin:glucagon ratio, it was determined that strong
relationships exist between NPY and both corticosterone and prolactin, but not the
insulin:glucagon ratio. There is a strong positive relationship between corticosterone and
NPY gene expression in the non-incubating group (Table A3.2). Analysis of variance
indicates that this relationship is significant. The data for the incubating group is confounded
by the small number of animals used, it appears though that four out of the five data points
correspond closely to the relationship observed in the non-incubating group (Figure A3.3).

For prolactin the results indicate a weak positive relationship with NPY expression in the non-
incubating group (Table A3.2) and a strong negative relationship in the incubating group.
Analysis of variance indicates that this relationship is significant. This raises the interesting
possibility that prolactin could have a role in regulating NPY’s effects on appetite that is
dependent upon the reproductive status of the animal. The insulin:glucagon ratio showed no
evidence of a relationship with NPY gene expression for either group.
Table A3.2: Correlation analysis comparing the expression of NPY relative to β-actin in incubating and non-incubating male emus with corticosterone and prolactin concentrations and the insulin:glucagon ratio.

<table>
<thead>
<tr>
<th>Group and hormone interaction</th>
<th>Correlation coefficient</th>
<th>F ratio</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-incubators – corticosterone (ng/ml)</td>
<td>0.994</td>
<td>256.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Incubators – corticosterone (ng/ml)</td>
<td>-0.692</td>
<td>2.759</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Non-incubators – prolactin (ng/ml)</td>
<td>0.538</td>
<td>1.63</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Incubators – prolactin (ng/ml)</td>
<td>-0.899</td>
<td>12.7</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Non-incubators – insulin:glucagon ratio</td>
<td>-0.542</td>
<td>0.53</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Incubators – insulin:glucagon ratio</td>
<td>0.469</td>
<td>0.85</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

Figure A3.3: Correlation analysis of the relationship between NPY gene expression relative to β-actin and corticosterone concentration (non-incubators = open circles and incubators = solid circles).
Question 6. Would differences have been more significant with greater n values?
If more animals had been available to use in the experiment the differences observed between the groups would have been more likely to be significant. Greater n values in each group would have reduced the confounding effects of the high variability observed between birds for many of the parameters measured.
Chapter 7.

Question 1. One week of starvation seems severe. Can this be justified? Did this and all experiments receive ethical approval beforehand?

The application of one week of starvation is justified given the much more extreme voluntary starvation that emus undergo during incubation. All experiments received approval from the UWA animal ethics committee and were performed in accordance with the animal ethics and welfare guidelines given by UWA. Chapter 2 has been amended to reflect this.

Question 2. Was the carcass analysis performed on the same subset of birds used for the hypothalamic NPY measurement?

Yes, the material and methods section of chapter 7 has been amended to more clearly reflect this.

Question 3. Another possibility worthy of discussion is that the RPA method of measuring mRNA in blocks of hypothalamic tissue fails to detect small but biologically significant changes in expression within specific nuclei, or in subpopulations of neurons within nuclei.

This is a fair criticism of the technique, for detecting genes that are weakly expressed or subtle changes in gene expression it is not an appropriate method unless the region where the gene is expressed is very accurately dissected. In Japanese quail fasted for 24 hours and chickens fasted for 48 hours or chronically feed restricted RPA detected for quail 1.5 and for chicken 2 fold increases in NPY gene expression (Boswell et al, 2002; Boswell et al, 1999; Boswell et al, 1999b). As such, RPA is a suitable technique for studying NPY expression in the emu hypothalamus.