STUDIES OF THE ENVIRONMENTAL AND ENDOCRINE CONTROL OF REPRODUCTION IN THE FOUR STRIPED FIELD MOUSE, *RHABDOMYS PUMILIO*.

THESIS

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ABSTRACT

Previous studies of the control of reproduction in *Rhabdomys pumilio* have shown that day length alone does not inhibit spermatogenesis, that a reduction in food availability and ambient temperature results in an inhibition of gametogenesis, that females are more susceptible to inhibition than are males, and that mice that are able to maintain a body fat store in the face of an energetic challenge, are less likely to show reproductive inhibition than those that lose their fat store.

In the present study, field and laboratory experiments were conducted to examine the effects of winter food supplementation on reproduction and population dynamics, and the effects of exogenous GnRH, leptin and mercaptoacetate (MA) on reproductive activity of *Rhabdomys pumilio* exposed to an energetic challenge. In the field food supplementation experiments in Thomas Baines Nature Reserve (2000, 2001), there was no winter inhibition of reproduction and provision of supplementary food had little effect. While at Mountain Zebra National Park (2002) winter was harsher, females became reproductively inactive, spermatogenesis continued and the provision of extra food resulted in higher rates of individual growth and larger reproductive organs.

Treatment of mice that had been exposed to a prolonged energetic challenge, with exogenous GnRH (1? g/mouse/treatment) resulted in an increase in the masses of the testes and epididymides, and in the activity of the reproductive organs. Treatment with exogenous leptin (40? g/mouse/treatment), concurrently with an energetic challenge, countered the negative effects of the energetic challenge, and treated males had larger reproductive organs. MA (600? mol/kg body mass), given concurrently with an energetic challenge, did not inhibit fat metabolism, although the high-fat diet countered the effects of the energetic challenge.

Results suggest that the first response of male *Rhabdomys pumilio* to an energetic challenge is a reduction in the size of the reproductive organs, without an inhibition of spermatogenesis. It is likely that this effect is mediated via white fat and leptin, and leptin's influence on the hypothalamic-pituitary-gonad axis. Results of the study support the suggestion that females are more sensitive to reproductive inhibition than males and that reproduction in *Rhabdomys pumilio* is truly opportunistic.

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CHAPTER ONE: INTRODUCTION – THE CONTROL OF REPRODUCTION IN RODENTS; THE ROLE OF ENVIRONMENTAL AND HORMONAL FACTORS.

Life histories of rodent species are highly variable in terms of the timing of reproductive activity (Zucker *et al.,* 1980; Neal, 1986). At one extreme, reproduction may be limited to a short breeding season, and at the other, reproduction may be continuous. Between these extremes there is a continuum of variation from a slight to an absolute tendency to concentrate the reproductive activity at a certain time or times in the year (Bronson & Perrigo, 1987; Bronson, 1989; Walkden-Brown *et al.*, 1994; White *et al.*, 1997). This variation in rodent life histories is seen both between species and within a species, and is often dependent on the environment in which the species or population is located (Bronson & Perrigo, 1987; Bronson, 1989).

Since rodents generally have short life spans, their reproductive activity is routinely dominated by the need to counter these short life expectancies (Bronson, 1989) by favouring a high rate of reproduction and, where necessary, rapid cessation of reproduction (Bronson & Heideman, 1994). However, factors other than longevity can influence reproductive activity. The diet of the species, whether it is a generalist or a specialist, and whether the quality and, or quantity of the food changes seasonally will affect the timing of reproduction (e.g. Perrin, 1980). The presence or absence of certain survival strategies, such as torpor or hibernation (Bronson, 1989), and a variety of environmental and social factors will act on the species (Martin, 1984). Environmental factors include climatic variability, food availability, latitude and altitude, while social factors include social interactions, which can indirectly affect predation pressure. Since the duration of the breeding season is dependent on climate and on food quality and quantity (Anderson & Meurling, 1977), it is not surprising that most mammals, including rodents, which live in environments where seasonal variation in the climate is associated with seasonal variation in food availability, reproduce seasonally (Bronson & Perrigo, 1987; Marsteller & Lynch 1987a). Such seasonal variation in climate and food availability is most obvious at temperate latitudes (Bronson, 1989; Demas & Nelson, 1998), where mammals, including rodents, will usually only breed during restricted and predictable periods in the year (Pryor & Bronson, 1981). However, as latitude decreases the variation in climate becomes less predictable and less extreme (Demas & Nelson, 1998), and the timing of reproduction becomes more flexible. Such flexible or opportunistic reproduction becomes increasingly evident at subtropical and tropical latitudes (Bronson, 1989; Bronson & Heideman, 1994; Bernard & Tsita, 1995). Seasonal reproduction, however, does occur at lower latitudes and is usually in response to seasonal changes in food abundance (Heideman & Bronson, 1990, 1993; Nelson *et al.*, 1997) rather than a direct response to climatic changes. However, such changes in food availability will often reflect changes in rainfall and/or temperature (Taylor & Green, 1976; Bronson & Perrigo, 1987; White *et al.*, 1997).

Ultimately, reproduction is regulated by energy availability, which itself is affected by ambient temperature and food availability, and is dependent on the energy cost/gain ratio of foraging (Marsteller & Lynch, 1987a; Bronson, 1989; I'Anson *et al.*, 1991; Heideman & Bronson, 1993; Bronson & Heideman, 1994). Reproduction is one of the most energetically costly activities, particularly for female mammals (Bronson & Heideman, 1994; Khokhlova *et al.*, 2000), and is reliant on the availability of metabolizable energy (Schneider & Wade, 1990a; I'Anson *et al.*, 1991; Wade *et al.*, 1996), and the presence of proteins, carbohydrates and fats in the diet (Taylor & Green, 1976; Wade *et al.*, 1996; Mattos *et al.*, 2000). Evidence for the importance of food availability has been provided by a number of studies which have indicated that, with a reduction in food availability alone, the maintenance and onset of gametogenesis is inhibited (Blank & Desjardins, 1984 for *Peromyscus maniculatus*; Bronson, 1989; Young *et al.*, 2000 for *Peromyscus leucopus*), and that a reduction in food during pregnancy will result in reduced fertility, an increase in pup mortality and a decrease in the weight of new-borns (see Bazhan *et al.*, 1996 for *Arvicola terrestris*; Marsteller & Lynch, 1987b). From these studies, it is clear that food availability can act as a proximate regulator as well as an ultimate cause of seasonal reproduction (Bronson, 1989; Bronson & Heideman, 1994).

The timing of reproduction can be fixed or modulated using predictive cues to restrict breeding to specific times of the year (Demas & Nelson, 1998). The use of such cues can allow animals to prepare themselves metabolically in advance of the time during which reproduction will be most profitable (Bronson & Perrigo, 1987; Bronson, 1989). However, the use of most predictors results in strictly seasonal reproduction and thus is not characteristic of small mammals that reproduce opportunistically (Bronson & Perrigo, 1987; Bronson, 1989). The best known predictor is daily photoperiod (Bronson & Perrigo, 1987; Bronson, 1989; Bronson & Heideman, 1994) and it is most widely used in the temperate and sub-polar regions, and, to a lesser degree, at the higher latitudes of the tropics (Heideman & Bronson, 1993; Bronson & Heideman, 1994). Below 30^o latitude the effect of photoperiod is reduced since the daily changes in day length are too small to enforce seasonal breeding (Bronson & Heideman, 1994; Bernard & Hall, 1995), and this cue is unlikely to be used in these regions. Since the response to photoperiod is variable, even at the higher latitudes, within and between species (Bronson & Heideman, 1994 for *Peromyscus* spp.; Demas & Nelson, 1998) and photoperiod is unlikely to be used at the lower latitudes (e.g. Bernard & Hall, 1995 for S*accostomus campestris*; Jackson & Bernard, 1999 for *Rhabdomys pumilio*), a variety of other environmental factors have been suggested as possible proximate cues for reproduction. These include temperature, rainfall and plant secondary compounds, acting either alone or in combination (Kenagy & Bartholomew, 1981; Marsteller & Lynch, 1987a; Bronner *et al.*, 1988; Bronson, 1989; Nelson *et al.*, 1992).

Ambient temperature will affect the thermoregulatory needs of a small mammal (Bronson & Perrigo, 1987; Bronner *et al.*, 1988; Bronson & Heideman, 1994), and this may compromise the energy available for reproduction. However low temperatures cannot be considered independently of food intake (Bronson & Heideman, 1994), since a reduction in temperature will have only a slight inhibitory effect on reproduction when food is abundant, while the inhibitory effects of reduced temperature will be exaggerated as food supply declines (Pryor & Bronson, 1981 for *Mus musculus*; Marsteller & Lynch, 1987a; Jackson & Bernard, 2001 for *Rhabdomys pumilio*). An exception to the inhibitory effects of low temperature is evident in desert animals, where it has been found that a reduction in temperature will enhance reproductive function, for example in *Perognathus formosus* (Kenagy & Bartholomew, 1981). High temperatures, probably functioning as a stressor, can affect reproduction and will do so independently of other controlling factors (Bronson & Heideman, 1994).

Rainfall is a limiting proximate factor that can influence plant productivity (Bronner *et al.*, 1988 for *Mastomys natalensis*) and therefore influence reproduction indirectly, or it can provide a source of water and thus affect reproductive activity directly, especially in desert and dry grassland animals (Kenagy & Bartholomew, 1981 for *Perognathus formosus*; Bronson, 1989;

I'Anson *et al.,* 1991). Related to rainfall, is the production of green plants and these can also stimulate reproduction through secondary plant compounds. The most commonly studied of these compounds is 6-methoxybenzoxazolinone (6-MBOA), which is considered a reliable cue indicating that the vegetative growing season has begun (Berger *et al.*, 1981; Linn, 1991; White *et al.*, 1997). A number of rodents have been shown to use this compound as a short-term cue, including *Microtus montanus* (Berger *et al.*, 1981; Sanders *et al.*, 1981; Bronson & Heideman, 1994), *Microtus pinetorum* (Schadler *et al.*, 1988), *Rattus norvegicus* (Butterstein *et al.*, 1985; Vaughan *et al.*, 1988), *Dipodomys ordii* (Rowsemitt & O'Conner, 1989), *Mus musculus* (Nelson & Shiber, 1990) and *Mastomys coucha* (Linn, 1991). However, not all rodents respond to this cue (White, 1999 for *Rhabdomys pumilio*; White & Bernard, 1999 for *Saccostomus campestris*). Secondary plant compounds, such as p-coumaric acid, may also be used to mark the end of the reproductive season (Batzli, 1983). P-coumaric acid is a phenolic compound produced by senescing plants and thus indicates the end of the growing season. However, exposure to p-coumaric acid did not negatively affect the reproductive organs of *Gerbillurus paeba* (White & Bernard, 1996).

As mentioned previously, social interactions can also influence reproduction and this occurs mainly via pheromones either in the urine of the rodents or as products of scent glands (Bronson, 1989). Pheromones produced by males can result in the stimulation of ovulation, hastening of sexual maturity and reduction of postpartum anoestrus in the female (Nelson, 1985 for *Microtus ochrogaster;* Bronson & Heideman, 1994; Rekwot *et al.*, 2001), and those produced by females may result in an enhancement of male arousal and sexual development (Bronson, 1989; Demas & Nelson, 1998 for *Peromyscus aztecus*; Rekwot, *et al.*, 2001), Alternatively, pheromones may inhibit the sexual maturation of same-sex individuals (Bronson & Heideman, 1994). Overall, social factors can regulate the seasonal onset and offset of breeding and can influence reproductive success during the breeding season (Bronson & Heideman, 1994).

All of these cues may play a role in the control of reproduction and rarely is only one cue responsible. Thus, there is generally an interaction of cues (Bronson, 1989; Bronson & Heideman, 1994; Heideman *et al.*, 1998) affecting the timing of reproductive activity. Small mammals from high temperate latitudes will typically be exposed to strongly seasonal and highly predictable environments and will mostly respond to photoperiodic cues, although other cues will also play a role, and reproduction will typically be seasonal (Trillmich, 2000). By contrast, small mammals in the tropics and subtropics may be exposed to a more stable and more predictable climate, will probably not rely on photoperiod as a cue, and may reproduce either seasonally or continuously. Of particular interest are intermediate areas, which may be characterised by a seasonal climate but a lack of predictability from one year to the next. In these areas, continuous reproduction may be possible in some years but not in others and this requires more flexibility in the control of reproduction than is necessary in other areas. In such areas, species or populations will usually respond opportunistically to favourable environmental conditions for breeding and will react to rainfall, temperature and food availability, with the possibility of modulation by social cues.

To add to the complexity of the control of reproduction, males and females of the same species may react differently to the various environmental and endocrine factors controlling reproduction (Pryor & Bronson, 1981; Bronner *et al.*, 1988; Wade *et al.*, 1996; Tilbrook *et al.*, 2000). This variation between genders may be related to the different energetic requirements of the female and male reproductive cycles (Bronner *et al.*, 1988). The oestrous cycle is an all-or-nothing event and is therefore likely to be more sensitive to energetic challenges than the males' reproductive cycle (Wood *et al.*, 1991; Bronson & Heideman, 1994; Nagatani *et al.*, 1996). In general, steroidogenesis in males is much more sensitive to energetic challenges than is spermatogenesis (Bronson, 1989; Nelson *et al.*, 1992; Bronson & Heideman, 1994). If food restriction is imposed early in life, before spermatogenesis has begun, it will greatly delay the onset of this process. However, once spermatogenesis has begun, food restriction will generally only inhibit spermatogenesis if it is severe and prolonged (Bronson, 1989; I'Anson *et al.*, 1991; Bronson & Heideman, 1994). In addition to this, it is known that only small amounts of testosterone and LH are necessary to maintain spermatogenesis (Bronson, 1989) while elevated levels are required to induce spermatogenesis (Handelsman *et al.*, 1999). It is also possible that the reason for the gender difference in the control of reproduction lies in the energetic costs of post-fertilization events. Female mammals carry all, or most, of the risks and energetic costs of successful fertilization (i.e. pregnancy and lactation) and its is thus not surprising that females appear to be more sensitive to reproductive inhibition than males (Bronson, 1989; Jackson & Bernard, 2001).

In view of these issues, and since spermatogenic cycles can take several weeks to complete, it is advantageous for males to remain reproductively ready for longer periods than females, in case the possibility of reproducing arises (Bronner *et al.*, 1988; Bronson, 1989; Bronson & Heideman, 1994). However, it is known that testosterone has a negative effect on the survival of males (Bousses & Chapuis, 1998); hence sexually active males are more prone to mortality than are inactive males (Bronson & Heideman, 1994). Therefore, there must be a trade-off between the costs and benefits of sexual activity and survival (Bousses & Chapuis, 1998) and in some instances the fertility of males may be reduced to lower levels or turned off completely until the next breeding season (Gerlach & Aurich, 2000). These gender differences are typical of an opportunistic breeding strategy and in its purest form it dictates that males remain sexually ready at all times of year (if beneficial to do so), and that females breed either seasonally or continuously, depending on the availability of energetic stores, independently of photoperiod (Bronson & Perrigo, 1987; Bronson & Heideman, 1994). In such cases, the breeding season would therefore be determined by the females of the species (Swanepoel, 1980; Clarke, 1985; Hamilton & Bronson, 1985; Bronner *et al.*, 1988), as has been noted in *Rhabdomys pumilio* (David & Jarvis, 1985), *Otomys irroratus* (Davis & Meester, 1981), *Apodemus sylvaticus* and *Clethrionomys glareolus* (Clarke, 1985).

The links between the environmental variables, which affect reproduction, and the endocrine system that ultimately controls reproduction are complex. The endocrine control of reproduction involves a hierarchy of endocrine organs comprising the hypothalamic-pituitary-gonad axis (HPG). At the top of this hierarchy is the hypothalamus from which stimulatory (or releasing) and inhibitory peptides are released. The synthesis and release of these peptides is regulated by neural networks and peptidergic pathways within the hypothalamus and other parts of the brain, which are influenced by environmental stimuli (Bronson, 1989; Dobson & Smith, 2000). The main peptide released by the hypothalamus is gonadotropin-releasing hormone (GnRH), which is released in periodic, short-lived bursts (Bronson, 1989) and is transported via a blood portal system to the anterior pituitary (see Bronson, 1989) where it stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH- McCann *et al.*, 1998; Marshall *et al.*, 2001). These two hormones then act on the gonads and promote the production of gametes and steroid hormones including testosterone, oestrogen and progesterone (Martin, 1984; Bronson, 1989). The production and release, and actions of GnRH, FSH and LH are in part influenced by feedback actions of the gonadal steroid hormones and inhibin (Martin, 1984; McCann *et al.*, 1998; Tilbrook *et al.*, 2000; Marshall *et al.*, 2001). Oestrogen increases GnRH release (Martin, 1984) and progesterone decreases GnRH release (Marshall *et al.*, 2001) thus affecting LH and FSH release accordingly; estradiol (produced by the ovary) enhances LH secretion (Marshall *et al.*, 2001); and inhibin inhibits the release of FSH from the anterior pituitary directly (see McCann *et al.*, 1998; Marshall *et al.*, 2001). In addition, a variety of other hormones and chemicals within the body can also influence the reproductive axis, either directly or indirectly. These include neuropeptide Y (NPY), opioids, corticotropin-releasing hormones, galanin, serotonin, catecholamines, cholecystokinin (CCK), insulin, growth hormone (GH), glucose, amino acids, proopiomelanocortin (POMC), leptin, gastric inhibiting peptide (GIP), nitric oxide (NO) and glucocorticoids (Barb *et al.*, 1982; I'Anson *et al.*, 1991; Wade *et al.*, 1996; Miller *et al.*, 1998; Cunningham *et al.*, 1999; Tilbrook *et al.*, 2000).

NPY is a potent orexigenic peptide that is expressed in the hypothalamus (Ahima *et al.*, 1996; Erickson *et al.*, 1996). It stimulates food intake, increases plasma insulin and corticosterone levels, decreases thermogenesis (Stephens *et al.*, 1995) and modulates GnRH release by inhibiting its secretion (I'Anson *et al.*, 1991; Cunningham *et al.*, 1999). NPY also inhibits the secretion of LH (McCann *et al.*, 1998; Clarke & Henry, 1999) and inhibits noradrenaline neurons (McCann *et al.*, 1998). Opioids are known to inhibit GnRH release (I'Anson *et al.*, 1991) and are affected by progesterone, which increases opioid activity in the hypothalamus (Marshall *et al.*, 2001) and by POMC, which results in the production of endorphin, an endogenous opioid (see Clarke & Henry, 1999). Corticotropin-releasing hormones, cytokines, CCKs and GIPs all suppress GnRH secretion and therefore the release of the gonadotropins (I'Anson *et al.*, 1991; McCann *et al.*, 1998). Glucose and insulin both act as metabolic cues affecting the release of GnRH and the gonadotropins (Estienne *et al.*, 1990; I'Anson *et al.*, 1991; Foster & Nagatani, 1999; Kittok, 1999). Glucose will maintain high levels of LH and FSH secretion (Estienne *et al.*, 1990; I'Anson *et al.*, 1991), as will insulin (Foster & Nagatani, 1999; Kittok, 1999). Prostaglandins and prostanoids both participate in the maturation of the reproductive system and both modulate GnRH release, mostly by stimulating its release, however some can inhibit the release of GnRH (Zhang *et al.*, 1992; McCann *et al.*, 1998).

Leptin (a hormone produced by adipose tissue) has been suggested as being a satiety factor in rodents informing the central nervous system about energy levels (Erickson *et al.*, 1996; Sansinanea *et al.*, 2001) and has been implicated in the regulation of a number of physiological processes including reproduction (Nagatani *et al.*, 1998; Clarke & Henry, 1999; Cunningham *et al.*, 1999). Leptin promotes sexual maturation in rodents and may affect the secretion of various hormones, either affecting reproduction directly or indirectly (Clarke & Henry, 1999). Leptin stimulates GnRH, LH and FSH secretion (McCann *et al.*, 1998; Nagatani *et al.*, 1998; Foster & Nagatani, 1999), reduces NPY activity (Stephens *et al.*, 1995; Ahima *et al.*, 1996), activates nitric oxide synthase (NOS – McCann *et al.*, 1998) and may change glucose availability (Nagatani *et al.*, 1998).

Nitric oxide (NO) has also been implicated in controlling reproduction. It is formed in the body by NOS, which converts arginine into citrulline and NO (McCann *et al.*, 1998; Dunnam *et al.*, 1999). NO promotes GnRH release (McCann *et al.*, 1998), inhibits ovarian hormone secretion and regulates steroidogenesis (Dunnam *et al.*, 1999). NO in turn is activated by glutamic acid and leptin (via NOS production) and is inhibited by gamma amino butyric acid and haemoglobin (McCann *et al.*, 1998).

A relationship between adrenal function and reproductive function has also been noted (see Barb *et al.*, 1982). Adrenocorticotropin (ACTH), which stimulates the release of glucocorticoids from the adrenal gland, blocks LH secretion and therefore affects ovulation (Barb *et al.*, 1982; Bronson, 1989), and cortisol (a glucocorticoid) interacts with oestradiol and reduces GnRH production (Tilbrook *et al.*, 2000). However, norepinephrine, which is also released from the adrenal gland, has stimulatory effects on the reproductive axis and stimulates the release of GnRH, NO and LH (McCann *et al.*, 1998).

From the presented information, it is clear that the endocrine control of reproduction is highly complex, involving a number of hormones and chemicals all interacting and affecting the HPG axis in various ways. How the environment interacts with the endocrine system is also complex, with different endocrine pathways being activated by the different external environmental factors. Most environmental factors influence the HPG axis primarily at the level of the hypothalamus by altering GnRH secretion (Nelson *et al.*, 1997; Miller *et al.*, 1998). Information about daily photoperiod is perceived by the pineal gland via the retina. In response to photoperiod, melatonin is secreted in the dark and affects GnRH production (Heideman & Bronson, 1990; Gerlach & Aurich, 2000). In short-day breeders, melatonin activates GnRH release, while it will inhibit the release of GnRH in long-day breeders (Gerlach & Aurich, 2000).

Nutritional effects are mainly mediated by changes in the activity of the hypothalamus, especially the GnRH neurons (I'Anson *et al.*, 1991; Wade *et al.*, 1996), although under-nutrition can affect all levels of the HPG axis (Wade *et al.*, 1996). Food deprivation as a whole will reduce the release of GnRH (Estienne *et al.*, 1990; Cunningham *et al.*, 1999; Foster & Nagatani, 1999; Kittok, 1999), resulting in a reduction in LH and FSH release (Sisk & Bronson, 1986; Cunningham *et al.*, 1999), therefore affecting reproductive activity directly. Food restriction can also affect the reproductive axis indirectly by enhancing the negative feedback sensitivity to the gonadal steroids (Bronson, 1989) and by affecting the pituitary-adrenocortical system (I'Anson *et al.*, 1991; Bazhan *et al.*, 1996; Dobson & Smith, 2000; Tilbrook *et al.*, 2000) where corticosterone levels are increased and cause a disruption of reproductive functions (I'Anson *et al.*, 1991; Bazhan *et al.*, 1996; Dallman *et al.*, 1999; Dobson & Smith, 2000). The lack of certain nutrients will also affect reproduction and in the case of glucoprivation, the action is mediated via the suppression of GnRH secretion from the hypothalamus (see Wade *et al.*, 1996; Cunningham *et al.*, 1999). Some nutrients have a stimulatory effect on reproduction, as is the case with fats in the diet, which will cause an increase in LH production, and cholesterol, which is used in the production of steroid hormones (Mattos *et al.*, 2000). Metabolic fuel availability will also affect the circulating levels of metabolic hormones such as insulin, GH, CCK and amino acids, all of which will affect the reproductive axis of an individual (see Wade *et al.*, 1996). In the same way that the pineal gland converts the day length message to an endocrine signal, so the white fat cells will convert a message about the size of the caloric store into an endocrine message through the production of leptin. While leptin generally conveys a stimulatory message to the HPG axis, NPY, produced by neurosecretory cells in the brain under conditions of starvation, conveys a negative message.

The majority of studies of the role of environmental variables and the endocrine system in the control of small mammal reproduction have been undertaken at high temperate latitudes where seasonal changes are dramatic and predictable. Based on the information presented above, there are good reasons to expect that the control of reproduction at lower latitudes will be quite different; species may respond to different cues and this may involve different neuroendocrine and endocrine pathways. Therefore, while we may have a quite good understanding of the control of reproduction in small mammals from temperate latitudes, this is not the case for species from lower latitudes. The aims of the research reported in this thesis were to use a combination of field and laboratory experiments to examine the environmental and endocrine control of reproduction in a small mammal from a seasonal but unpredictable environment. Specifically, experiments have examined the effects of food supplementation in the field on the reproduction and population dynamics during winter; the effects of exogenous GnRH on the reproductive activity of male *Rhabdomys pumilio* exposed to low ambient temperatures and low food availability; the effects of exogenous leptin on the reproductive activity of male *Rhabdomys pumilio* exposed to low ambient temperatures and low food availability; and the role of fat metabolism and its effects on reproductive activity in male *Rhabdomys pumilio*.

CHAPTER TWO: *RHABDOMYS PUMILIO* IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA.

The aim of this chapter is to introduce the study animal, providing some information on its biology, and to provide information on the climate of the study areas.

THE STUDY ANIMAL:

The four striped field mouse (*Rhabdomys pumilio* (Sparrman, 1784) Rodentia, Muridae), is a small, diurnal rodent that is characterised by four black stripes running down its back from the head to the base of the tail (Figure 2.1). The mean adult body mass ranges from 30 to 50g (although pregnant females can weigh as much as 80g) and there is no sexual dimorphism (De Graaff, 1981; David & Jarvis, 1985; Yom-Tov, 1993; Jackson & Bernard, 1999).

Figure 2.1: Typical adult, four striped field mouse, *Rhabdomys pumilio* showing the characteristic stripes on its back.

Rhabdomys pumilio occurs throughout most of Africa, south of the Sahara (Figure 2.2; De Graaff, 1981; David & Jarvis, 1985) and has been studied in Kenya (Taylor & Green, 1976), Malawi (Hanney, 1965), Botswana (Smithers, 1971) and South Africa. The most extensive study on the ecology of this rodent in South Africa, was a field study conducted by Brooks (1974) in Van Riebeeck Nature Reserve, Pretoria. Other South African field and laboratory studies include those by Perrin (1980) in the Eastern Cape Province; Henschel *et al.* (1982) and David & Jarvis (1985) in the Western Cape Province, and Jackson & Bernard (1999, 2001) in the Eastern Cape Province. *Rhabdomys pumilio* occurs mainly in grassland where cover is thick, although it does occur in other areas such as thickets, where clumps of thick grass or bush are available for protection (Taylor & Green, 1976; Dewsbury & Dawson, 1979; Yom-Tov, 1993; pers. obs.). The four striped field mouse is a generalist feeder, mainly taking seeds but it will also eat insects, fruit and green vegetable matter (Perrin, 1980; Brooks, 1982; David & Jarvis, 1985; Koshy, 2002).

Rhabdomys pumilio is considered to be of economical and medical importance. It is one of the first colonisers of recently disturbed (burnt or cleared) areas (van Hensbergen *et al.*, 1992) and is often attracted to cultivated lands, where it is known to cause damage to young trees (Davis, 1942; Koshy, 2002), grain crops and especially grain stores. *Rhabdomys pumilio* is also known to be a vector of a number of viruses and parasites (Brooks, 1974) including the plague bacillus (*Pasteurella pestris*), nematodes, cestodes and ticks. Although *Rhabdomys pumilio* is not currently an economic or medical pest in South Africa, it is a pest in other regions of its distribution, for example in East Africa where it attacks maize and wheat crops (Taylor, 1968; Liers, 1999).

Figure 2.2: Map of Africa indicating the distribution of *Rhabdomys pumilio* (shaded areas) based on the available literature. The actual distribution patterns of the mice within the countries are not known and therefore the whole country is shaded. (Map modified from WorldAtlas.com.)

The timing of reproductive activity of *Rhabdomys pumilio* varies through Africa. In Malawi, it has been reported that they are seasonal breeders (Hanney, 1965). In Botswana, *Rhabdomys pumilio* has been reported to breed during two periods of the year, January to February and June to July (Smithers, 1971), while in Kenya, they show opportunistic tendencies, breeding when conditions are favourable (Taylor & Green, 1976). Although Dewsbury and Dawson (1979), who worked on mice collected from Kenya, have reported continuous reproduction in *Rhabdomys pumilio* in captivity, they support the theory of seasonal breeding in these mice in the wild. In South Africa, a number of studies have reported seasonal breeding in *Rhabdomys pumilio* (Brooks, 1974 – Pretoria; Perrin, 1980 – Eastern Cape Province; Henschel *et al.*, 1982; David & Jarvis, 1985 – Western Cape Province). However David and Jarvis (1985) reported finding a small number of reproductively active males and females during the supposed non-breeding season (May to August). More recently, Jackson and Bernard (1999) collected a pregnant female and some spermatogenically active males during winter (May to September) in the Eastern Cape Province of South Africa. Based on these published records, it was suggested that *Rhabdomys pumilio* reproduces opportunistically and exhibits seasonal reproduction in response to climatic and dietary changes during the year (Jackson & Bernard, 1999).

Rhabdomys pumilio can produce large litters, ranging in size from 2-9 neonates, over a short period of time (Dewsbury & Dawson, 1979; Brooks, 1982; David & Jarvis, 1985; Jackson, pers. obs.). They have a short gestation period of approximately 25 days and an early weaning age (14 days) (Dewsbury & Dawson, 1979; David & Jarvis, 1985). There is disagreement over how many litters an individual mouse can produce within a year, with suggestions ranging from 3 to 7 in the wild (Perrin, 1980; David & Jarvis, 1985) and 14 in captivity (Dewsbury & Dawson, 1979).

A number of studies have been conducted to establish the factors that control reproduction in *Rhabdomys pumilio*. Photoperiod, which is often a major controlling factor in the temperate latitudes of America and Europe (Bronson, 1989), has been tested and short-day length alone does not inhibit spermatogenesis (Jackson & Bernard, 1999). It has been suggested that rainfall may stimulate reproductive activity (Perrin, 1980). However, field reports have shown that this is unlikely, since in the Western Cape Province (34^{o}00'S, 18^{o}35'E) *Rhabdomys pumilio* breeds during the summer months, while the majority of the rainfall in this region falls during the winter months (David & Jarvis, 1985). It has been shown that a reduction in ambient temperature and/or food availability, resulting in an energetic challenge, can inhibit reproductive activity. However, these effects vary with gender and body fat content (Jackson & Bernard, 2001).

THE STUDY AREA:

The study was conducted in the central region of the Eastern Cape Province of South Africa (Figure 2.3), which has a strongly seasonal climate characterised by a warm to hot, wet summer and colder, drier winter. Conditions at the coast are milder than those experienced in the interior regions. In the Grahamstown and the Cradock areas, the mean minimum temperatures between May and September are below 11°C, and these months represent winter (Bernard *et al.*, 1991).

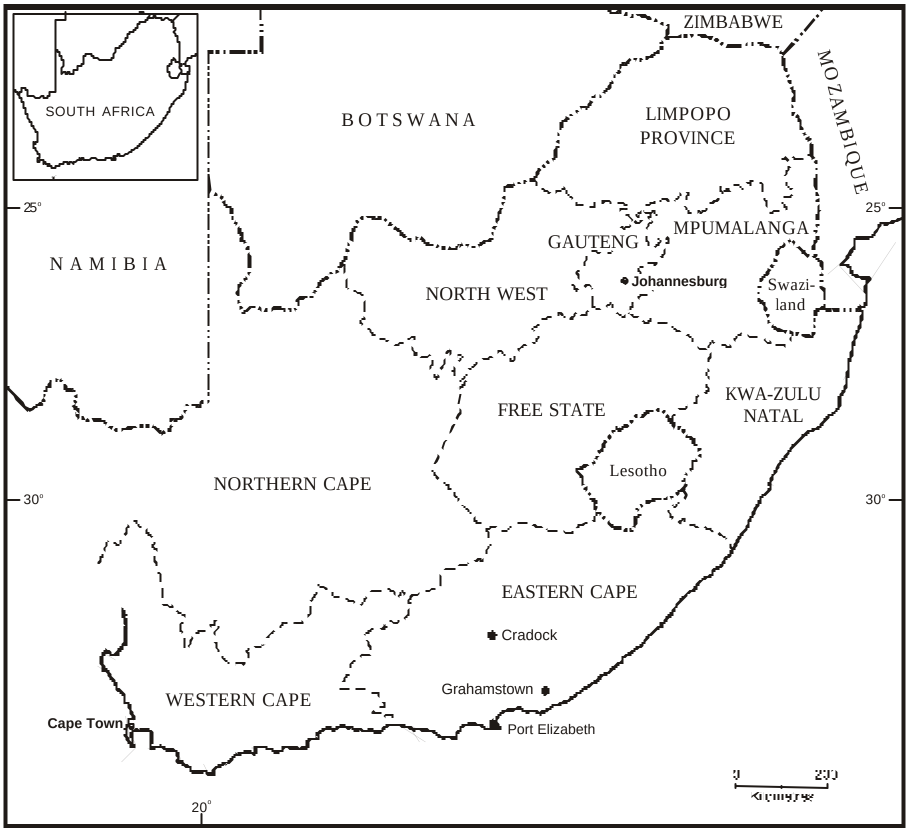

Figure 2.3: Map of South Africa (insert) showing the location of the provinces and the two study areas (Grahamstown and Cradock) within the Eastern Cape of South Africa. (Map courtesy of Rhodes University Graphics services.)

Due to South Africa being influenced by El Niño and La Niña (the counterpart to El Niño), the rainfall in South Africa is highly variable. During the years when El Niño influences this country, rainfall tends to be lower than average, with cooler temperatures during the summer months. When La Niña influences South Africa, the opposite is usually true, with above average rainfall and warmer temperatures occurring both during summer and winter (South African Weather Service, 2001a & b). In addition to the effects of the El Niño Southern Oscillation (ENSO - includes La Niña), the Eastern Cape Province is situated on a transition zone between a summer rainfall region (KwaZulu-Natal Province) and a more mediterranean, winter rainfall region (Western Cape Province). With both of these factors acting upon the Eastern Cape Province, the rainfall and seasonal temperatures in this region are unpredictable, resulting in some years having warmer and wetter winters and drier, hotter summers than usual.

Within the Eastern Cape Province, two study areas were used. Thomas Baines Nature Reserve, which is located 10 kms south east of Grahamstown (33°18'S 26°32'E; Figure 2.3), is the region from which all of the mice used in the laboratory experiments were caught. This reserve was also the site of two of the food supplementation field experiments (see Chapter 3). During summer (October to April) the mean monthly maximum temperatures for this region remain above 21°C while the mean monthly minimum temperatures rarely fall below 10°C (Figure 2.4). Winters (May to September) are mild with mean monthly maximum temperatures remaining between 17°C and 23°C, although the area can experience mean monthly minimum temperatures as low as 6°C. Although the pattern of seasonal temperature change in this region (Grahamstown and surrounding areas) tends to remain stable from one year to the next (Figure 2.4), some variations do occur. The pattern of rainfall for Grahamstown shows marked inter-annual variation (Figure 2.5). Generally, Grahamstown experiences its maximum rainfall during the summer, however in 1997, June (winter) had the second highest rainfall for that year and the amount of rain that fell was about 20 times greater than is usually experienced in the winter months (1994-1999).

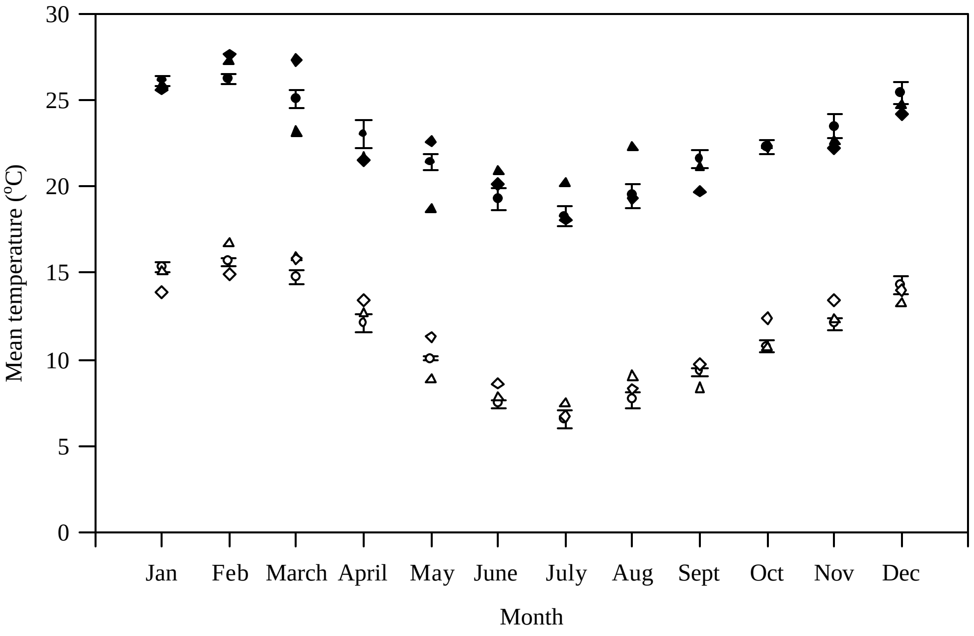

Figure 2.4: Mean (? 1SEM) temperatures collected for Grahamstown from 1994 to 1999 (circles) with the mean monthly temperatures for 2000 (triangles) and 2001 (diamonds). Minimum temperatures = clear symbols; maximum temperatures = closed symbols.

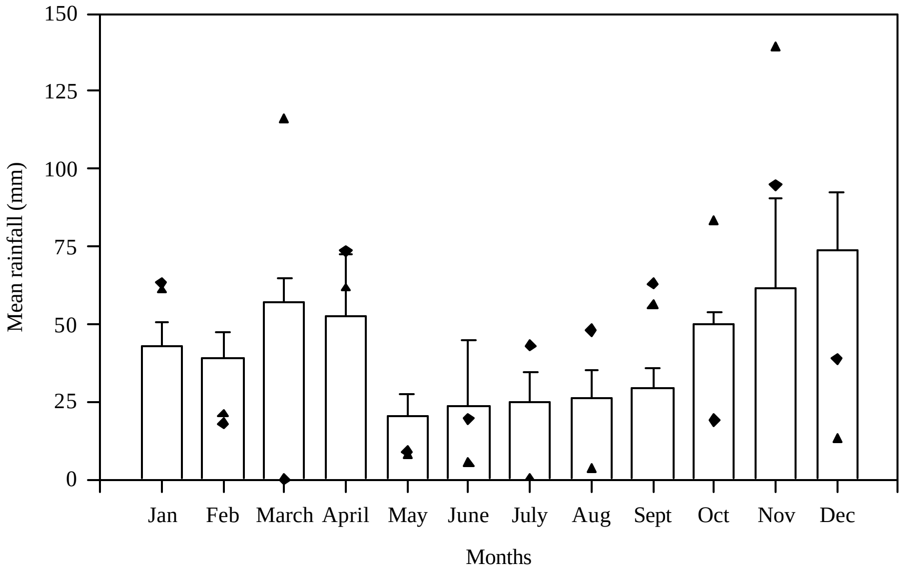

Figure 2.5: Mean (? 1SEM) monthly rainfall for Grahamstown for the years 1994 – 1999 (Histogram bars) with the mean rainfall for 2000 (triangles) and 2001 (diamonds).

The second study site was in Mountain Zebra National Park (MZNP) which is located just outside Cradock (32^{o}11'S 25^{o}37'E), and lies 200 km inland of Grahamstown (Figure 2.3). Based on climatic data collected from 1994 to 2000, the Cradock area has less inter-annual variation than Grahamstown, although it does experience greater variation in seasonal temperatures (Figure 2.6). During summer (October to April) the mean monthly maximum temperatures remain above 25^{o}C with a slight decrease in April, while in winter (May to September) the mean monthly maximum temperatures remain between 15^{o}C and 22^{o}C. The mean monthly minimum temperatures for summer do not fall below 10^{o}C, but in winter the mean monthly minimum temperatures remain below 8^{o}C and can get as low as 1.8^{o}C (July, 1997). The rainfall in this region is more seasonal compared to Grahamstown, with the winter months being drier with less than 30mm falling in any one month (May to August) and the summer months receiving between 19 and 60mm on average. Most of the inter-annual variation in rainfall pattern tends to occur within the summer months (Figure 2.7 & 2.8), with little variation occurring during winter.

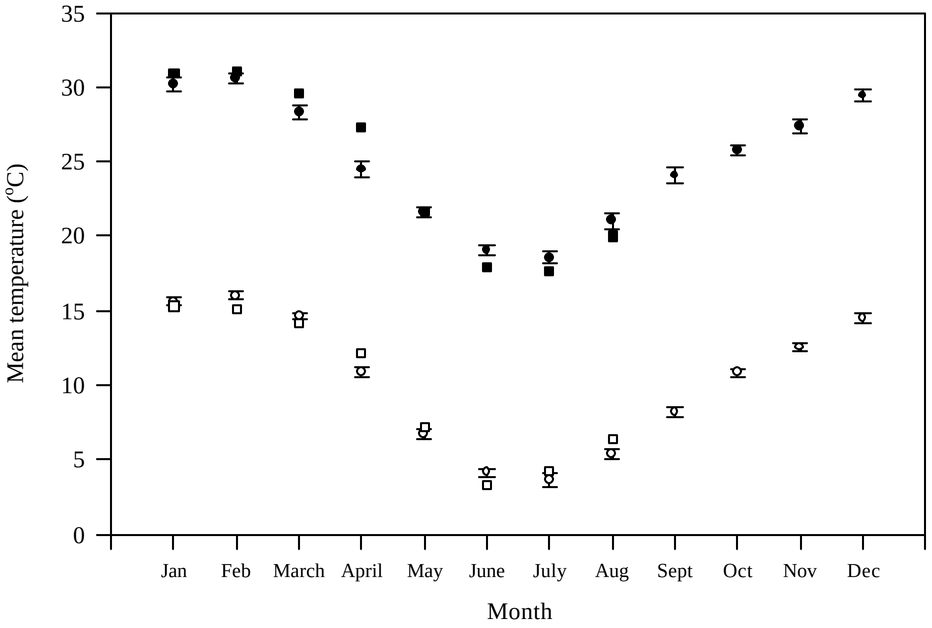

Figure 2.6: Mean (? 1SEM) temperatures collected for Cradock from 1992 to 2001 (circles) with the mean monthly temperatures for 2002 (squares). Minimum temperatures = clear symbols; maximum temperatures = closed symbols.

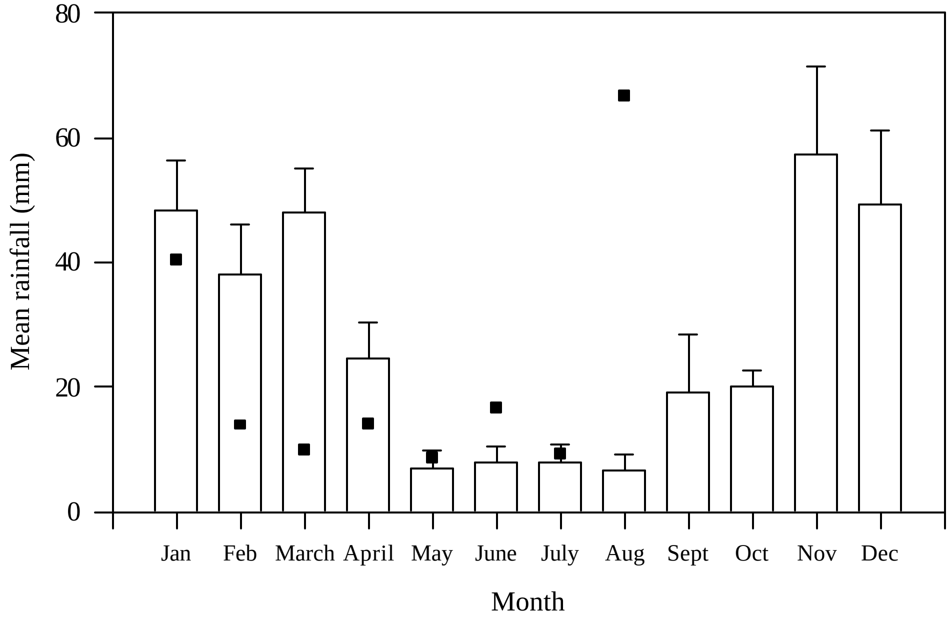

Figure 2.7: Mean monthly (? 1SEM) rainfall for Cradock area from 1992 to 2001 (histogram bars) with the monthly rainfall for 2002 (closed squares).

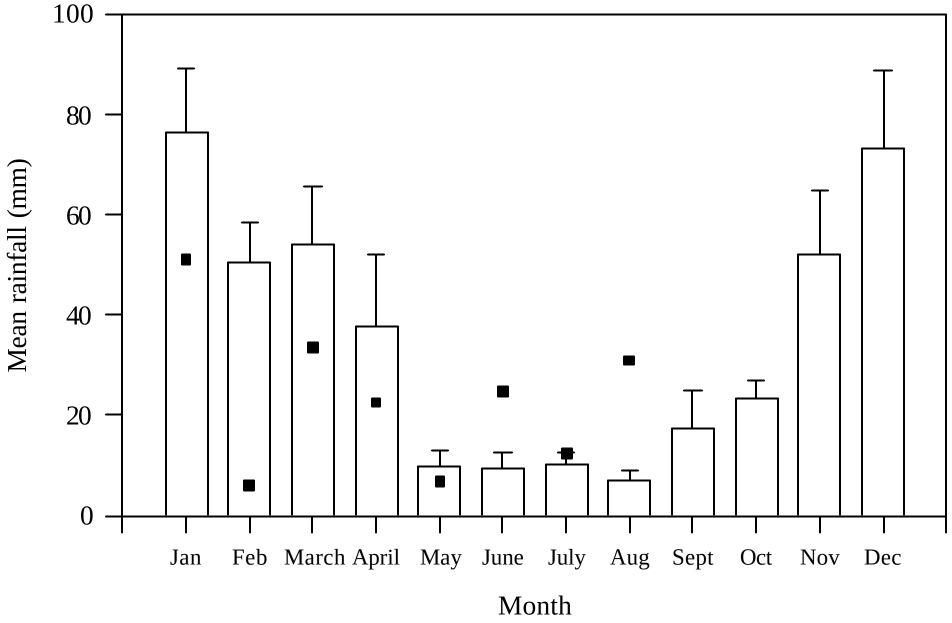

Figure 2.8: Mean monthly (? 1SEM) rainfall for Mountain Zebra National Park from 1992 to 2001 (histogram bars) with the monthly rainfall for 2002 (closed squares).

In summary, the two study areas are characterised by seasonal changes in temperature and rainfall. These seasonal changes are greater and are more predictable in MZNP than the climatic changes occurring in Thomas Baines Nature Reserve.

CHAPTER THREE: EFFECTS OF FOOD SUPPLEMENTATION ON REPRODUCTIVE ACTIVITY AND POPULATION DYNAMICS OF *RHABDOMYS PUMILIO*, IN THE FIELD DURING WINTER.

Food has long been considered to be one of the major environmental factors that influences population dynamics in rodents (Saitoh, 1989; Bronson & Heideman, 1994), whereby a reduction in food supply can limit population densities (Lack, 1954 – cited by Cittadino *et al.*, 1994; Briggs, 1986) and reduce reproductive traits, including breeding intensity, length of breeding season, litter size and increase the age at first reproduction (O'Donoghue & Krebs, 1992; Galindo-Leal & Krebs, 1998; McAdam & Millar, 1999). Due to this effect of food on reproduction and population dynamics, a number of field studies have been conducted to determine the effects of food supplementation on populations of various mammal species, including voles (e.g. Taitt & Krebs, 1983; Saitoh, 1989), squirrels (e.g. Sullivan, 1990; Nunes *et al.*, 2000) and hares (e.g. Vaughan & Keith, 1981; O'Donoghue & Krebs, 1992). Such experiments have tested whether or not an increase in food availability will result in: (1) an increase in reproduction such as an advancement and extension of the breeding season, higher reproductive output, winter breeding and an increase in the number of pregnant females; (2) an increase in survival of individuals; (3) an increase in population densities; and (4) an increase in immigration into the supplemented area, resulting in an increase in overall population size (Hansen & Batzli, 1979; Desy & Thompson, 1983; Briggs, 1986; Cittadino *et al.*, 1994; Monadjem & Perrin, 1996). Other hypothesised effects of the provision of supplementary food include an increase in the body mass of individuals and increased individual growth rates (Havera & Nixon, 1980; Desy & Thompson, 1983; O'Donoghue & Krebs, 1992).

These studies have shown that the provision of supplementary food either during or after the breeding season resulted in: an increase in the body mass of the experimental animals; an increase in the densities of the populations provided with food, mainly through immigration; an increase in the number of individuals that were reproductively active; an advancement and extension of the breeding season; an improvement in survival of both adults and non-adults; and an improvement in physical condition, growth rate and body fat content (*Peromyscus leucopus* – Hansen & Batzli, 1979 and Briggs, 1986; *Sciurus niger* and *Sciurus carolinensis* – Havera & Nixon, 1980; *Microtus pennsylvanicus* – Desy & Thompson, 1983; *Lepus americanus* –

O'Donoughue & Krebs, 1992; *Akodon azarae* – Cittadino *et al.*, 1994; *Praomys natalensis* – Monadjem & Perrin, 1996).

Recently it was shown in a laboratory study that a combination of low ambient temperature and reduced food availability inhibits gametogenesis in *Rhabdomys pumilio* (Jackson & Bernard, 2001) and that food availability has the greater effect. Based on these results, it was suggested that what is seen as seasonal reproduction (winter inhibition of reproduction) is a result of seasonal changes in climate and food quality and, or quantity (Jackson & Bernard, 2001). The most direct way to test this suggestion is to provide additional food in the field in winter and to assess the effect of this on reproduction in *Rhabdomys pumilio*. Thus, a series of experiments were conducted whereby populations of *Rhabdomys pumilio* were supplied with supplementary food during the winters of three consecutive years (2000 - 2002). The reproductive status and dynamics of these populations were assessed and the data were compared to those collected from populations that had not received supplementary food.

Since the food supplementation experiments were conducted over three years at two different study sites and with minor differences in methodology, each experiment will be presented separately with an overall discussion at the end.

FOOD SUPPLEMENTATION EXPERIMENTS: THOMAS BAINES NATURE RESERVE

STUDY SITE:

The study site for the first two food supplementation experiments in 2000 and 2001 was located in Thomas Baines Nature Reserve. The study site was established in a flat, open space in an area of Eastern Thorn Bushveld (Low and Rebelo, 1996), which was dominated by grasses, *Acacia karroo* and low bush (*Rhus pallens*; Figure 3.1). Since the study site was situated in a nature reserve, it was exposed to the presence of large, medium and small mammals and various bird species. Large to medium mammals included: white rhinoceros (*Ceratotherium simum*), buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*), warthog (*Phacochoerus aethiopicus*), common duiker (*Sylvicapra grimmia*), vervet monkey (*Cercopithecus pygerythrus*), baboon (*Papio ursinus*), scrub hare (*Lepus saxatilis*), impala (*Aepyceros melampus*), while the smaller mammals in the area included the pygmy mouse (*Mus minutoides*), vlei rat (*Otomys irroratus*), four striped field mouse (*Rhabdomys pumilio*) and pouched mouse (*Saccostomus campestris*). The climate for this region has been described in Chapter 2.

Figure 3.1: Study site for the food supplementation experiments conducted in Thomas Baines Nature Reserve during the winters of 2000 and 2001. Note the mixture of grasses, small shrubs and small *Acacia karroo* trees (see arrows).

FIELD EXPERIMENT 2000:

3.1) Methods:

3.1.1) *Field data collection*:

Within the study site, two trapping grids were established such that the grids were 100m apart. Although this distance was great enough to reduce the occurrence of mice crossing over from one grid to the other, the grids were close enough to reduce the likelihood of differences in climate and vegetation. Each grid consisted of 50 traps set out in a 5x10 manner, with individual traps being 10m apart. Half way between the two grids, a single row of traps was set to monitor any movement of mice from one grid to the other. These traps were set every 5m and were specifically orientated so that half the traps faced the one grid and the other half faced the second grid. Sherman traps, baited with peanut butter and oat balls, were used throughout the study and were placed under grass clumps or bushes to provide some protection from climatic extremes.

The experimental period started in early May 2000, at the start of winter, and ran for three months, finishing in early August. At the start of the experiment and for four days during the second week of each month, a trapping session was carried out, during which the traps were checked six times. Traps were opened and baited on the morning of day one and were checked twice a day, starting on the afternoon of day one and finishing in the morning of day four.

When specimens of *Rhabdomys pumilio* were caught, the trap number in which they were caught was recorded, the weight of the animal was taken and the length of its hind foot was measured. Each mouse was sexed, aged and its reproductive status was noted. Males were recorded as being either non-scrotal (testes abdominal), partly-scrotal (testes moving between abdominal position and scrotal position) or scrotal (testes in scrotal sacs). Females were noted as being either perforate (vulva open) or non-perforate (vulva closed). However, if the female was non-perforate but showed signs of being pregnant or of lactating, she was considered to be reproductively active, despite the closed vulva. Individuals were then marked by hair clipping according to the grid on which they were caught, for example on the left rump for the control grid and on the right rump for the supplemented grid. The position of marking was changed each month so as to prevent confusion, since the clipped hair may take more than a single month to regrow. After recording the data and noting whether the individual was a new capture or a recapture, the mouse was released at the trap station where it had been caught. Mice were aged according to their size, weight and coat appearance. Juveniles were less than 25g in weight and had a spiky coat and were reproductively inactive; sub-adults were medium sized, ranging from 25g to 35g in weight, had smooth, dark coloured coats and were reaching sexual maturity; and adults were medium to large in size, weighed more than 35g, had smooth, light coloured coats and were sexually mature. The separation of sub-adults and adults was difficult at times because there was some variation in the size at which individuals became sexually mature. The use of mass as part of the classification provided a more objective separation of these two age groups. The choice of 35g to separate sub-adults from adults was supported by work by Brooks (1982), who indicated that in a population of *Rhabdomys pumilio*, 50% of the population was sexually mature at a mass of 30g while the whole population had reached sexual maturity at 40g. It is important to note that in this study, sub-adults were considered capable of becoming sexually mature and therefore reproductively active.

Weights (to the nearest 0.5g) were taken using a hand-held spring balance (Super Samson, 200g/2g) with a plastic bag attached to the hook of the balance (Figure 3.2) and hind foot measurements (to the nearest mm) were taken using a pair of callipers.

Mice that were caught in the midline traps were examined for markings, and if present, measurements were taken as described above. These mice were considered to be foraging and were released on the grid from which they came. However, if the mouse was unmarked, it was released at the trap site without any data being collected. If a mouse from one grid was trapped on the other grid (as noted by their clipped fur), it was remarked and considered as an immigrant and therefore released on the grid to which it had moved.

Figure 3.2: Method used for weighing mice in the field, using a hand held spring balance attached to a plastic bag with the mouse (*Rhabdomys pumilio*) inside.

After the first trapping session (May), the grid on the western side of the study site was allocated as the supplementation grid. Within this grid, two petri dishes (9cm in diameter) per trap site were filled with mixed bird seed and placed close to the trap (n = 100 feeding stations). The dishes were hidden under bushes or clumps of grass to prevent birds from seeing them and consuming the food. Experiments in the laboratory had shown that *Rhabdomys pumilio* readily consumes bird seed. For the rest of the experimental period, food was placed into the dishes every second or third morning, and some food was scattered around the trap area (broadcast feeding), such that food was always available on the grid. On each occasion that food was provided, dishes were examined for signs of seed consumption and for the presence of faeces.

Throughout the three months, minimum and maximum ambient temperatures were noted on the days that supplementary food was provided and on the days of trapping, using a min/max thermometer that was left hanging in a tree within the study area. The amount of food distributed on the supplemental grid was also recorded.

In the final trapping period (August) adult mice were collected from both grids and were brought into the laboratory.

3.1.2) *Sacrifice, tissue collection and blood collection:*

At the end of the August trapping session, adult mice were brought into the laboratory. These mice were sacrificed by administration of 0.2ml of Euthanaze (sodium pentobarbitone, Centaur Labs, Bryanston), weighed, dissected and given a subjective fat index from 0-3, which was based on the amount of fat around the reproductive organs, the kidneys and under the skin (0 = no fat present, 1 = fat present in one of these areas, 2 = fat present in two of the areas and 3 = fat around the reproductive organs, kidneys and under the skin; Jackson & Bernard, 2001). The hind foot length was also noted. The testes, epididymides and accessory glands (seminal vesicles and coagulatory glands) were removed from the males, and the ovaries and uterine horns were removed from the females. These reproductive organs were weighed to the nearest 0.001g and prepared for histological analysis using standard procedures. Blood was collected from the heart by ventricular puncture, centrifuged (13 750 rpm) for 10 minutes, and the plasma collected and stored at -10^{o}C.

3.1.3) *Histological analysis:*

Reproductive organs were prepared for histological analysis using standard methods (Carleton & Drury, 1962). Wax blocks were sectioned using a Leica microtome at 10?m and ribbons of sections were stained using Mallory's trichrome stain (see appendix for recipe and protocol). Slides were examined under a compound light microscope (Nikon).

3.1.3.1) Males:

Spermatogenic activity was assessed by examining 100 sections through seminiferous tubules per animal and scoring each section as spermatogenically active if spermatozoa or spermatids were present (1) or inactive (0) if there were only spermatogonia, primary spermatocytes and sertoli cells present. For each animal, the score was then expressed as a percentage and for each group of mice a mean value was calculated. A mouse was considered to be spermatogenically inactive if less than 25% of the tubules examined were spermatogenically active, and active if over 70% of the tubules examined were spermatogenically active (Jackson & Bernard, 2001). In most cases the division between spermatogenically inactive and active animals was clear and in active animals, 80-100% of the seminiferous tubules had spermatozoa, while in inactive animals, less than 25% of the seminiferous tubules had spermatozoa. Mice with intermediate values (25-70%) were considered to be becoming spermatogenically inactive if the experiment was designed to switch spermatogenesis off, or becoming spermatogenically active if the experiment was designed to turn spermatogenesis on. For each animal, twenty sections through the cauda epididymides were examined and the amount of sperm present in the sections was given a subjective score where 0 = no sperm in storage, 1 = less than $^{1}/_{3}$ of the area of the lumen filled with sperm, 2 = $^{1}/_{3}$ to a $^{1}/_{2}$ of the tubule full of sperm, and 3 = more than $^{1}/_{2}$ full. The mean score per animal was then expressed as a percentage of 60, which is the maximum score an animal could receive if all 20 sections of the cauda epididymides were full of sperm. The volume of secretary material stored in the seminal vesicles was assessed in a similar way based on twenty sections through the accessory glands (Jackson & Bernard, 2001).

3.1.3.2) Females:

Serial sections were cut through the centre of each ovary of each female. Because, on occasion, an ovary was either damaged or lost in processing, the sections from one ovary of each female were examined and the numbers of secondary follicles, Graafian follicles and corpora lutea within that ovary were recorded. The corpora lutea were also assessed for the extent of vascularisation present and were given a subjective score ranging from 0 to 3 (0 represents no vascularisation, 1 = few small areas of vascularisation, 2 = larger areas of vascularisation, and 3 = large areas vascularised and central cavity full of blood). The greatest diameter of the largest corpus luteum was also measured. One section through the wall of each uterine horn of each female was examined and the total thickness of the uterine wall, and the thicknesses of the endometrium and the myometrium were measured at two places on opposite sides of the uterine horn. The total number of uterine glands was counted in the same sections and the vascularisation of the uterine wall was scored subjectively (0 = no vascularisation, 1 = slight vascularisation, 2 = vascularisation around the uterus but with small vessels, 3 = large amounts of vascularisation and large blood vessels in the uterine wall). All measurements of histological slides were made with an optical micrometer (Filar, Nikon) and values were converted to the nearest 0.01?m.

3.1.4) *Hormone assay*:

Testosterone levels were determined using a Coat-a-count Total Testosterone kit (Diagnostic Products Corp. USA), with human serum-based calibrators. The limit of detection (95%) binding was 4ng/dL and inter- and intra-assay coefficients of variation for two serum samples included in each assay were less than 6% and 15%, respectively. Serial dilution of plasma yielded dilution curves that were not different from those obtained with the hormone standards.

3.1.5) *Population and statistical analyses*:

Estimates of population sizes during the experiment were obtained using mark-recapture data and the Lincoln index/ Peterson estimate (Caughley, 1977). The Lincoln index uses the equation: $N = Mn/m$, where the number of animals in the population (N) is related to the number of individuals marked and released (M), multiplied by the number of mice caught at a subsequent time (n) and divided by the number of mice recaptured at that time (m). In this study the index was slightly modified whereby the captures and recaptures over the four-day trapping session were pooled (Schumacher's method – in Caughley, 1977).

Statistical analyses were conducted using SigmaStat (Jandel Scientific, 1994), Statgraphics (Manugistics, 1992) and Statistica (Statsoft, Inc., 2001). Field data (number of mice caught and reproductively active) were compared using Log-linear analysis of frequency tests. Mass, foot length and dissection data were compared using student t-tests, analyses of variance (ANOVA), multiple analyses of variance (MANOVA) and the non-parametric equivalents where applicable. Percentages were arcsine transformed for statistical analysis, but these data are represented as percentages within figures and tables. Differences were considered significant at $a < 0.05$ and values are expressed as means ± 1 SEM, unless otherwise stated.

3.2) Results:

3.2.1) *Climate:*

Data from the South African Weather Service indicated that, with the exception of May, winter 2000 was milder and drier than previous years (1994-1999; Figures 2.4, 2.5). The minimum and maximum temperatures recorded at the study site were variable during the experiment and were at their highest during June (Figure 3.3, Table 3.1). Mean monthly minimum and maximum temperatures recorded in the field (Table 3.1) were higher than is usually experienced in this region (see Figure 2.4). The mean monthly maximum temperature varied significantly (P = 0.003) and the mean maximum temperature for June was significantly higher than maximum temperatures for both May and August (Table 3.1). The mean minimum temperatures did not vary significantly (P = 0.103). Little rain fell in the Grahamstown region during the winter of 2000 (see Figure 2.5).

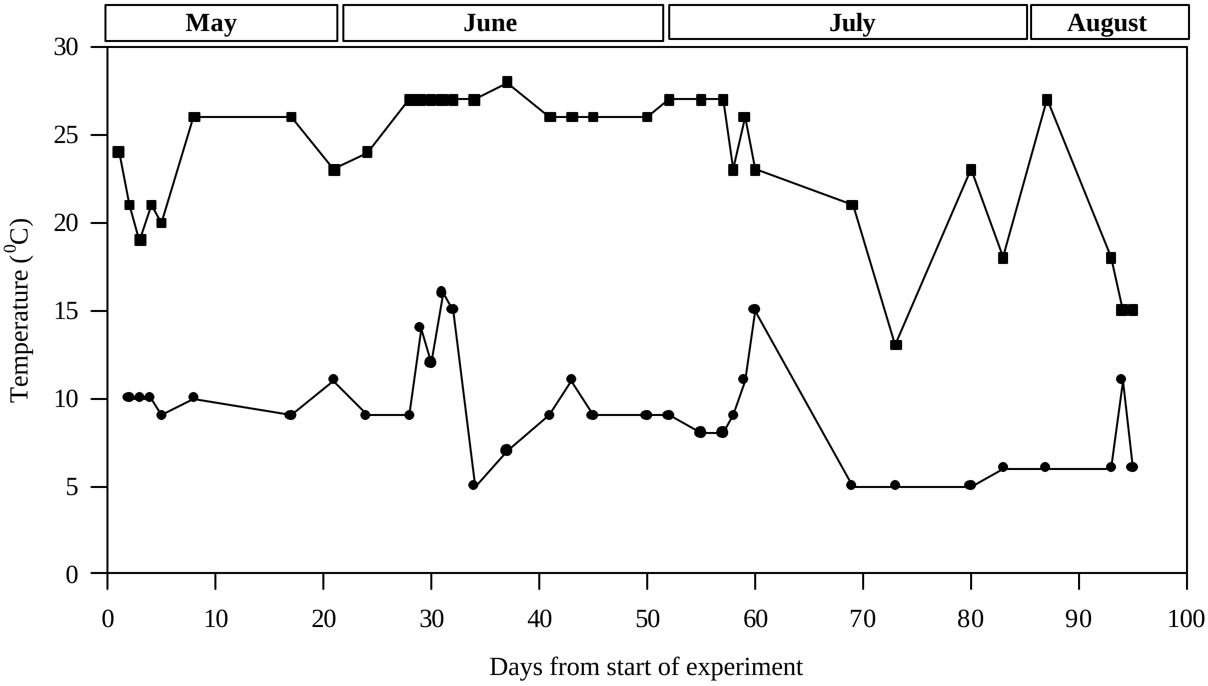

Figure 3.3: Minimum (circles) and maximum (squares) temperatures recorded in the field at times of feeding and trapping in Thomas Baines Nature Reserve during the winter 2000 food supplementation experiment.

Table 3.1: Mean monthly maximum and minimum field temperatures for the winter 2000 food supplementation experiment. Values are means ? 1SEM.

Month	Minimum temperature	Maximum temperature
May	9.86 ± 0.26^{a}	22.5 ± 0.95^{a}
June	10.31 ± 0.88^{a}	26.5 ± 0.27^{b}
July	8.00 ± 1.12^{a}	$22.3 \pm 1.52^{a,b}$
August	7.25 ± 1.25^{a}	18.8 ± 2.84^{a}

- Within a column, groups with different superscript letters (a or b) are significantly different ($P<0.05$).

3.2.2) *Field data:*

During the experiment 92kg of food was dispensed on the supplemented grid. While dispensing the food, it was noted that faeces, similar to those produced by *Rhabdomys pumilio*, were found in the petri dishes and it was therefore assumed that *Rhabdomys pumilio* were utilising this food source.

Results from the mark-recapture estimates indicated that the population on the supplemented grid in June and July was significantly larger than the population on the control grid. On both grids, the estimates of population size significantly increased from May to July ($P < 0.001$; Table 3.2).

Table 3.2: Estimated population sizes of *Rhabdomys pumilio* on two grids in the winter 2000 field supplementation experiment. Note that food supplementation began after the May trapping session and that mice were removed from the population during the August trapping session, hence estimates for this month have not been calculated. (See Table 3.3 for total number of first captures).

	Control grid	Supplemented grid
May	11	15
June	20	45
July	31	63
August	-	-

During the experiment five mice (three from the supplemented grid; two from the control grid) moved from one grid to the other, however once on the new grid they remained in the region for the rest of the experiment. A maximum of four marked mice were trapped in the midline per trap session and these mice were returned to the grid on which they were first marked.

Dealing with the number of mice caught (ignoring recaptured mice), it was evident that fewer mice were caught during May on both grids, and that the numbers of first-time captures increased significantly from the start of the experiment to the end, with maximum captures occurring in July and August (Tables 3.3, 3.4). In terms of age structure, significantly more adults were captured than juveniles and sub-adults on both grids ($P < 0.001$), except in May when more juveniles ($n = 7$) were caught on the control grid than adults ($n = 4$) and sub-adults ($n = 0$) on the same grid. No juveniles were caught on the control grid in August. The supplemented grid yielded significantly more captures during the experiment than the control grid ($P = 0.013$). There were significantly more reproductively active (scrotal and partly scrotal males, perforate and pregnant/lactating females) mice on the supplemented grid ($P = 0.005$), although there was no significant change in the number of reproductively active mice over the experimental period on either of the two grids ($P = 0.063$; Tables 3.3, 3.4).

Table 3.3: Population dynamics of *Rhabdomys pumilio* trapped on the control and supplemented grids in Thomas Baines Nature Reserve during the winter 2000 food supplementation experiment. Numbers are of individual mice, excluding recaptures.

	Control Grid				Supplemented Grid			
	May	June	July	August	May	June	July	August
Number of mice caught:								
Juveniles	7 (3? ; 4?)	7 (7?)	10 (6? ; 4?)	0	2 (2?)	6 (4? ; 2?)	3 (2? ; 1?)	2 (2?)
Sub-adults	0	1 (1?)	5 (3? ; 2?)	9 (4? ; 5?)	7 (2? ; 5?)	7 (4? ; 3?)	8 (2? ; 6?)	7 (4? ; 3?)
Adults	4 (1? ; 3?)	9 (2? ; 7?)	13 (12? ; 1?)	18 (12? ; 6?)	8 (3? ; 5?)	18 (8? ; 10?)	25 (12? ; 13?)	25 (12? ; 13?)
Total caught	11	17	28	27	17	31	36	34
% mice per trap session:								
Juveniles	64	41	36	0	12	19	8	6
Sub-adults	0	6	18	33	41	23	22	20
Adults	36	53	46	67	47	58	70	74
% mice reproductively active per age group per trap session:								
Juveniles	0	0	0	0	0	0	0	0
Sub-adults	0	100	0	0	57	14	0	14
Adults	75	67	77	61	63	84	72	48
Total N^{o} reproductively active	3	7	10	11	9	17	18	13

Table 3.4: Results of log-linear analysis of frequency tests conducted on the population field data collected during the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve.

	Comparison	**P-value**
No. mice caught	Months	P = 0.001
	Ages	P < 0.001
	Grids	P = 0.013
No. reproductively active	Months	P = 0.063
	Grids	P = 0.005

MANOVAs conducted to test the effects of grid and month on body mass and hind foot length of each age group, indicated that month had a significant effect on the body masses of each of the age groups ($P < 0.05$), where juveniles and sub-adults were significantly heavier in June and adults were significantly heavier in May than in any other month. There was a significant effect of month on the foot length of adults ($P < 0.001$), where adults were significantly larger in August than in May (Figure 3.4; Table 3.5). Grid (i.e. provision of supplementary food) had no significant effect on body mass or hind foot length (Table 3.5).

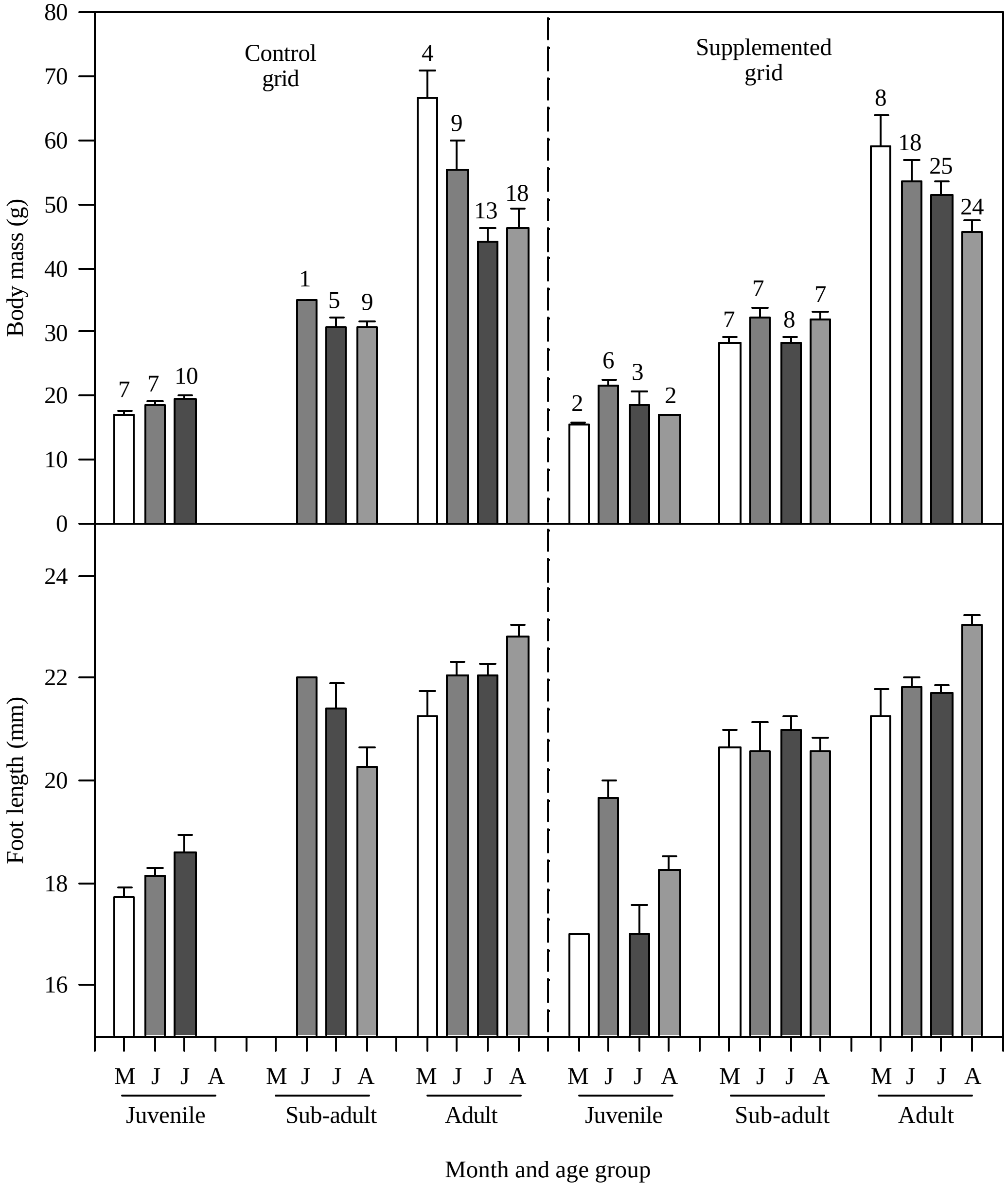

Figure 3.4: Mean body mass and hind foot length of mice trapped during the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Mice are classified according to their age group and the grid on which they were caught, and according to the month in which they were trapped. M, J, J, and A represent the months May, June, July and August respectively. Means are ? 1SEM. The numbers above the bars represent the sample size per group.

Table 3.5: Results of MANOVAs conducted on the mean body mass and mean hind foot length of mice from two grids for the various months in which mice were caught, during the winter 2000 food supplementation experiment conducted in Thomas Baines Nature Reserve.

Age group	Month	Grid	Month x Grid
Body mass			
Juvenile	P = 0.006	P = 0.373	No interaction
Sub-adult	P = 0.036	P = 0.556	No interaction
Adult	P < 0.001	P = 0.804	No interaction
Hind foot length			
Juvenile	P = 0.055	P = 0.965	No interaction
Sub-adult	P = 0.292	P = 0.656	No interaction
Adult	P < 0.001	P = 0.658	No interaction

Division of all mice caught in each month into nine weight classes indicated that during May, the control grid was dominated by mice of low mass (10.0 to 29.9g) with only four mice falling into the higher weight categories (50.0-79.9g; Figure 3.5). The mice on the supplemented grid were more evenly spread through the weight classes with two peaks occurring in the 20.0-29.9g size class and 50.0-59.9g size class, both of which were dominated by females (Figures 3.5, 3.6). In June, there were two main weight classes on the control grid (10.0-19.9g and 40.0-49.9g), the former being dominated by the males and the latter by females (Figures 3.5, 3.6). Mice on the supplemented grid weighed between 10.0g and 89.9g, with a mode at 30.0-39.9g (Figure 3.5).

In July, none of the mice were heavier than 70g (Figure 3.5). The majority of the mice on the control grid weighed less than 50g, while the majority of mice on the supplemented grid weighed between 20.0 and 69.9g. In August, both grids were characterised by a peak in the 30.0-39.9g weight class which was dominated by males on the control grid and females on the supplemented grid (Figures 3.5, 3.6).

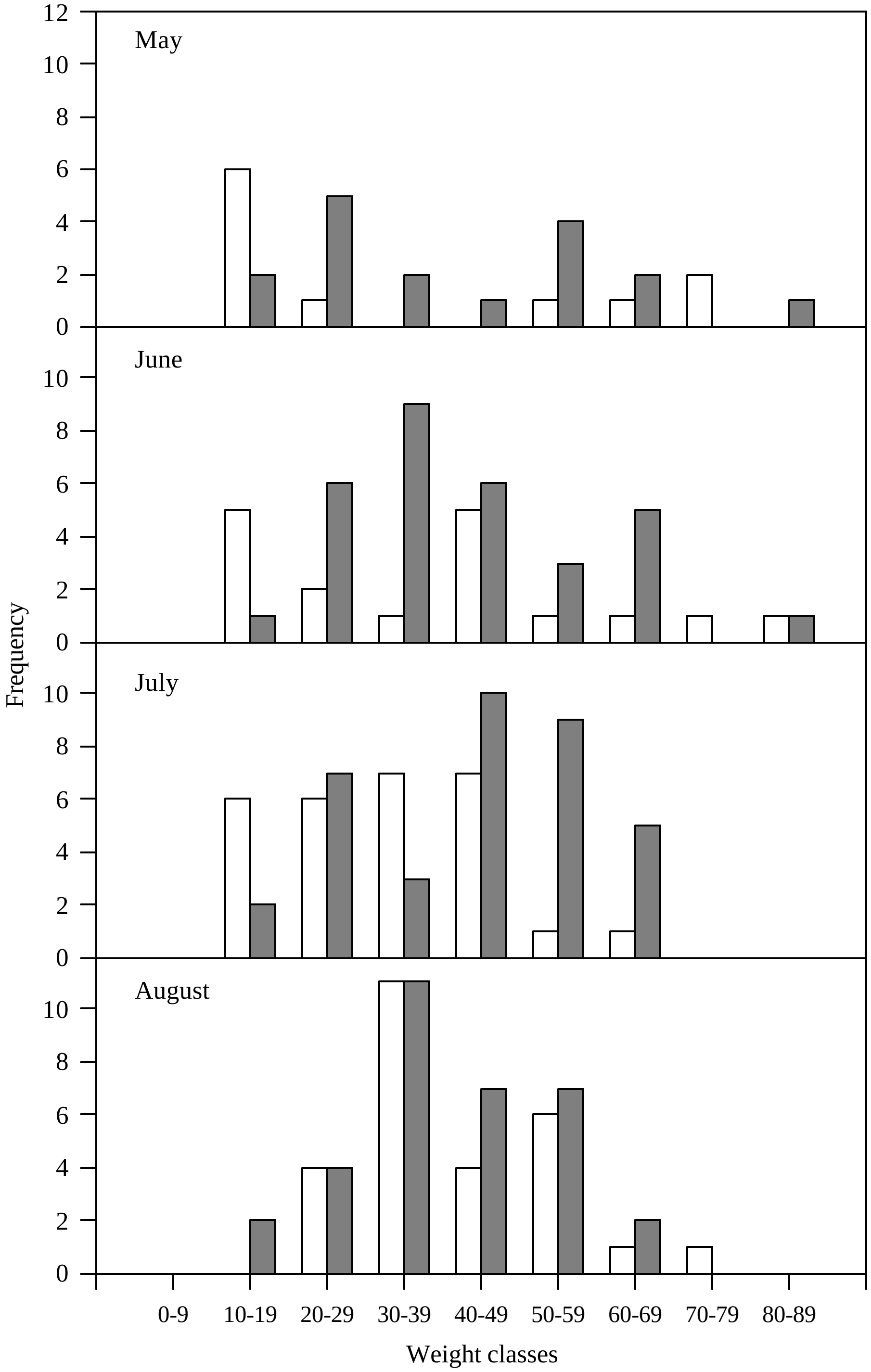

Figure 3.5: Size distributions of mice from the control and experimental grids of the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Weight classes are in grams and tick labels have been rounded to save space (0-9g = 0.0-9.9g). Clear bars denote the control grid; shaded bars denote the supplemented grid.

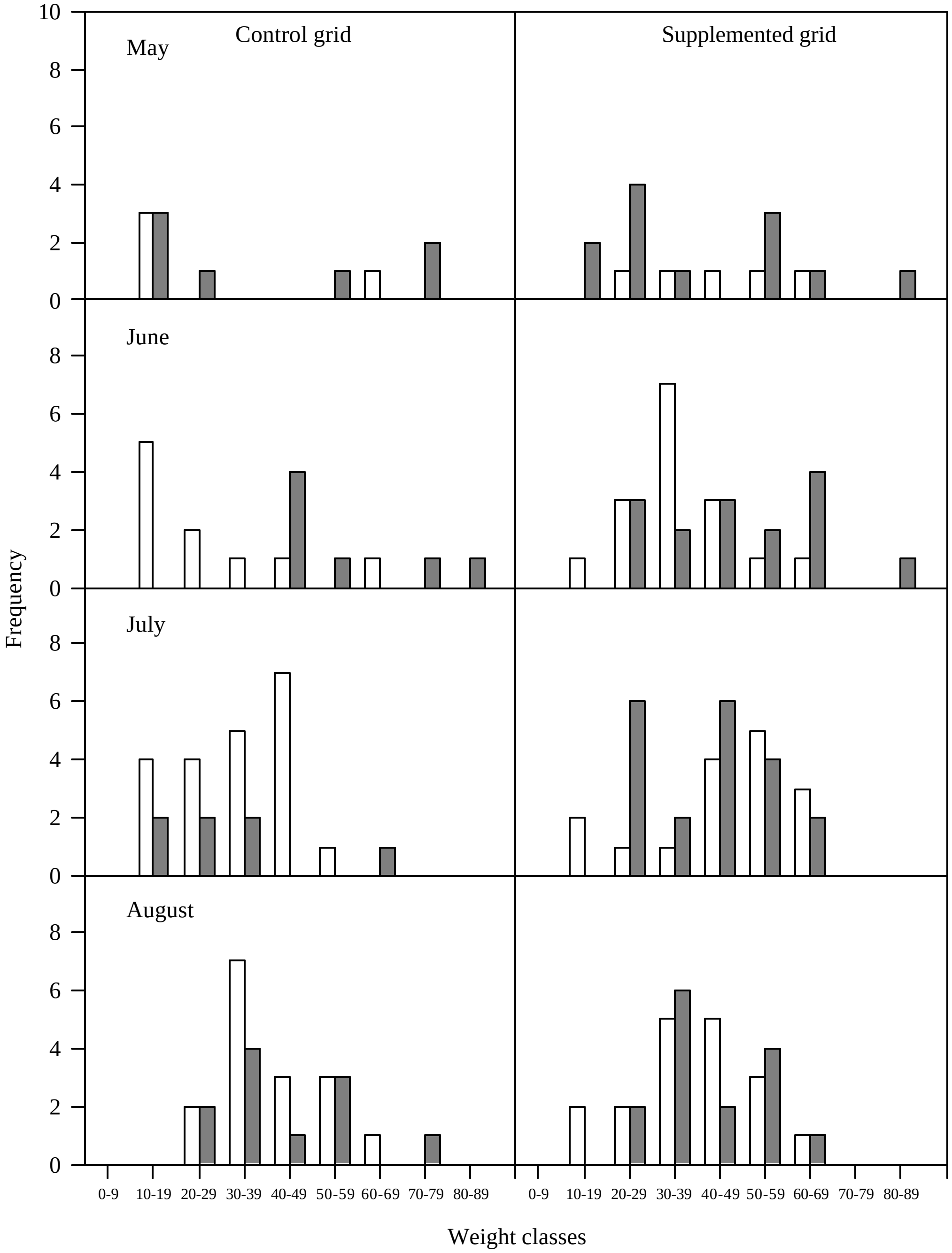

Figure 3.6: Size distributions of mice from the control and experimental grids of the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Weight classes are in grams and tick labels have been rounded to save space (0-9g = 0.0-9.9g). Clear bars denote males; shaded bars denote females.

A MANOVA conducted on the mean body masses of the mice (Table 3.6) from the two grids for each of the months indicated that there was a significant effect of grid on the body mass (P = 0.028), where the mice on the supplemented grid were heavier than those on the control grid, especially in June and July. There was no significant effect of month (P = 0.481) nor gender (P = 0.059) on the mean body masses of the mice, nor was there a significant interaction between month, grid and gender (P = 0.473).

Table 3.6: The mean body masses (g) of the males and females and the overall mean body mass for the mice on the control and experimental grids during the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Values are means ? 1SEM. (See Table 3.3 for sample sizes).

	Means	**May**	**June**	**July**	**August**
Control grid	Total	35.1 ? 7.66	39.1 ? 5.04	33.0 ? 2.35	41.0 ? 2.46
	Female	36.0 ? 9.66	54.9 ? 5.92	29.0 ? 5.79	42.4 ? 4.51
	Male	28.5 ? 10.84	28.0 ? 5.24	34.3 ? 2.49	40.1 ? 2.82
Supplemented grid	Total	41.3 ? 4.81	42.6 ? 3.08	43.6 ? 2.47	41.0 ? 2.06
	Female	39.8 ? 6.25	48.9 ? 5.19	42.1 ? 3.11	40.8 ? 2.77
	Male	45.0 ? 7.21	36.8 ? 2.92	45.6 ? 4.01	37.8 ? 2.95

3.2.3) *Dissection data:*

At the end of the experiment a total of 35 (10 males and nine females from the supplemented grid; 10 males and six females from the control grid) mice were caught and brought into the laboratory for dissection. Of the females captured for dissection, three from the supplemented grid and four from the control grid were pregnant. Litter sizes of pregnant females ranged from 4 to 6 neonates and there was no significant difference between the two grids (P = 0.206). On the supplemented grid seven of the males were non-scrotal and three were partly-scrotal, while four of the males from the control grid were non-scrotal, four were partly-scrotal and the remaining two males were scrotal. There was no significant difference in the reproductive status of mice from the two grids (P > 0.05). At dissection, there was no significant difference in the mean body masses or hind foot lengths between the males and females from the two grids (P > 0.05 for both; Figure 3.7A & B). Mice on the two grids had similar mean body fat scores (P > 0.05), varying in score from 0 to 2 (Figure 3.7C). The mean masses of the reproductive organs of both the males and females were not significantly different across the grids (P >0.05; Figure 3.7D & E).

3.2.4) *Histology:*

All the males from the two grids were spermatogenically active and there was no significant difference in the mean percentage spermatogenic activity of the males from the two grids (P = 0.762; Figure 3.8). Similarly, food supplementation did not have a significant effect on the storage of sperm in the epididymides or the activity of the accessory glands (P > 0.05; Figure 3.8). Although there were differences in the size and number of the ovarian follicles, and size and development of the uterine horns of females from the two grids (Figures 3.9 – 3.12), none of these were statistically different (P > 0.05 for all).

3.2.5) *Hormone assay:*

Mean plasma testosterone levels of the males did not differ significantly between the two grids (P = 0.857; supplemented grid, ? = 38.1 ? 20.3ng/dL; control grid, ? = 211 ? 116.3ng/dL).

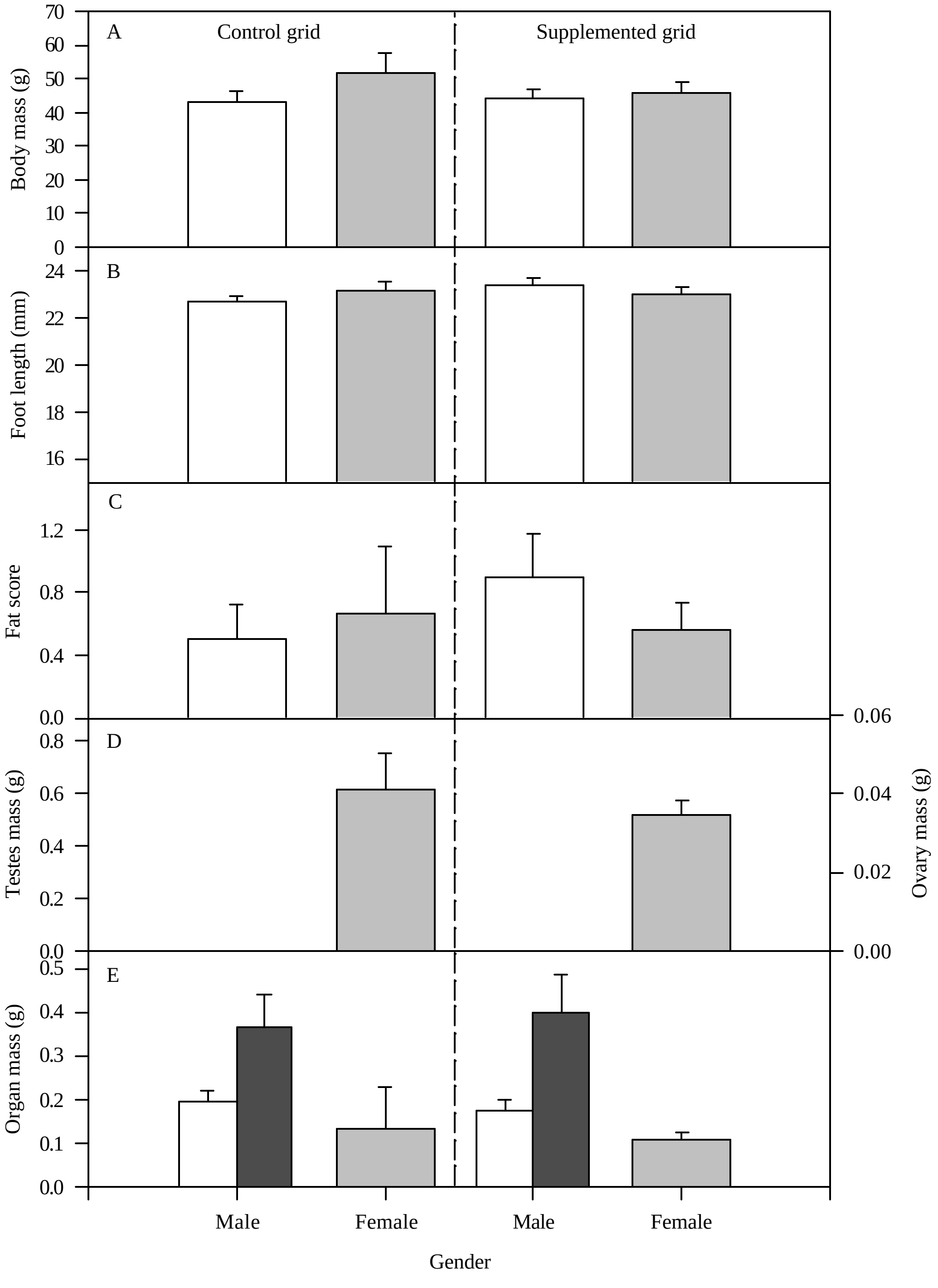

Figure 3.7: Dissection data for mice collected after the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve, indicating (A) mean body mass; (B) mean hind foot length; (C) mean fat score; (D) mean mass of the testes and ovaries; and (E) the mean mass of the epididymides (clear bars), accessory glands (cross-hatched bars) and uterine horns (dark bars). Values are means ? 1SEM.

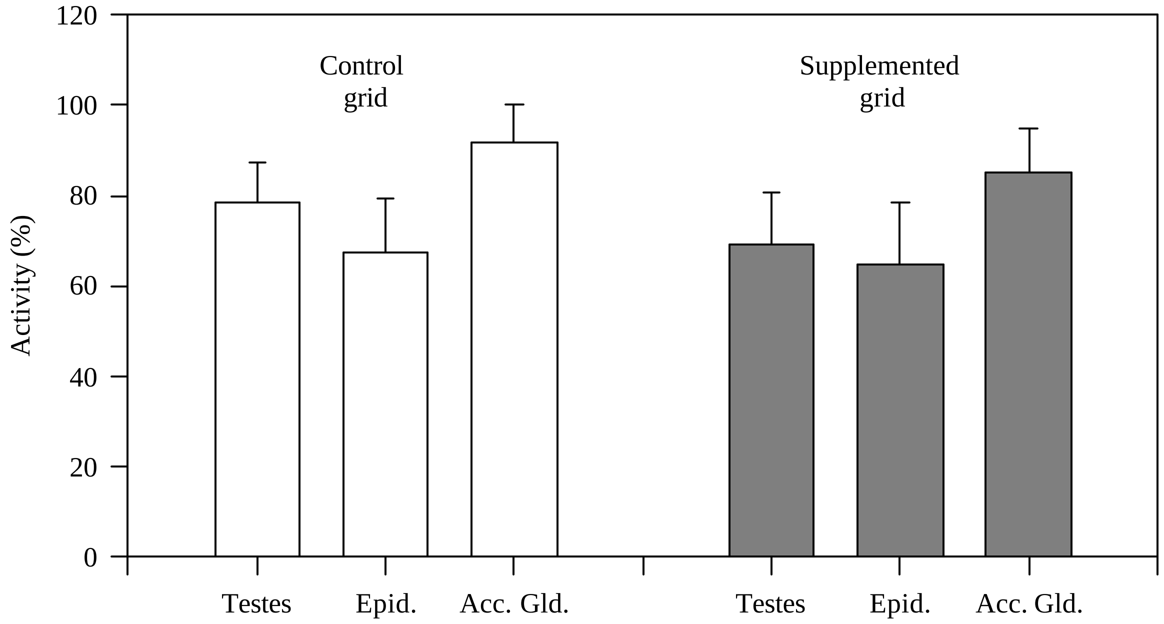

Figure 3.8: Mean activity (%) of the reproductive organs collected from male *Rhabdomys pumilio* after the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Epid = epididymides; Acc. Gld. = accessory glands. Values are means ? 1SEM.

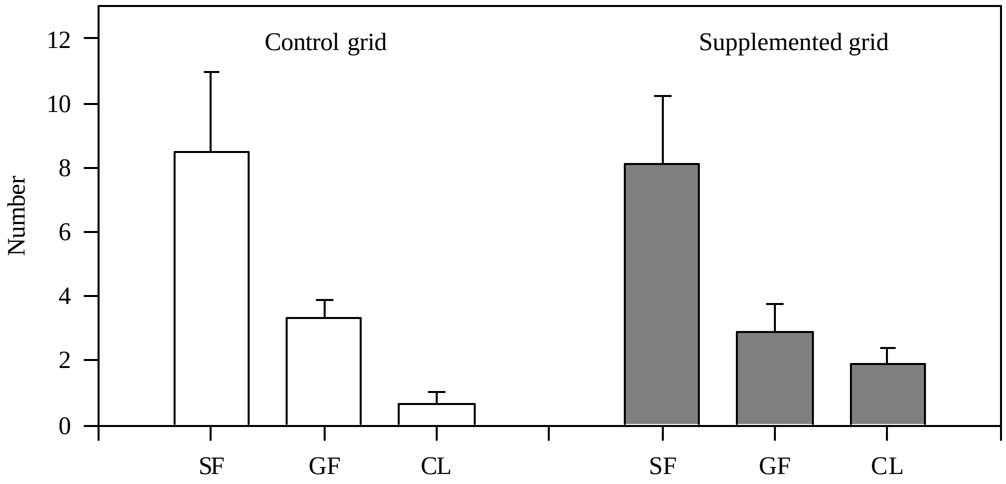

Figure 3.9: Mean number of secondary follicles (SF), Graafian follicles (GF) and corpora lutea (CL) from one ovary per individual female *Rhabdomys pumilio* caught after the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Values are means ? 1SEM.

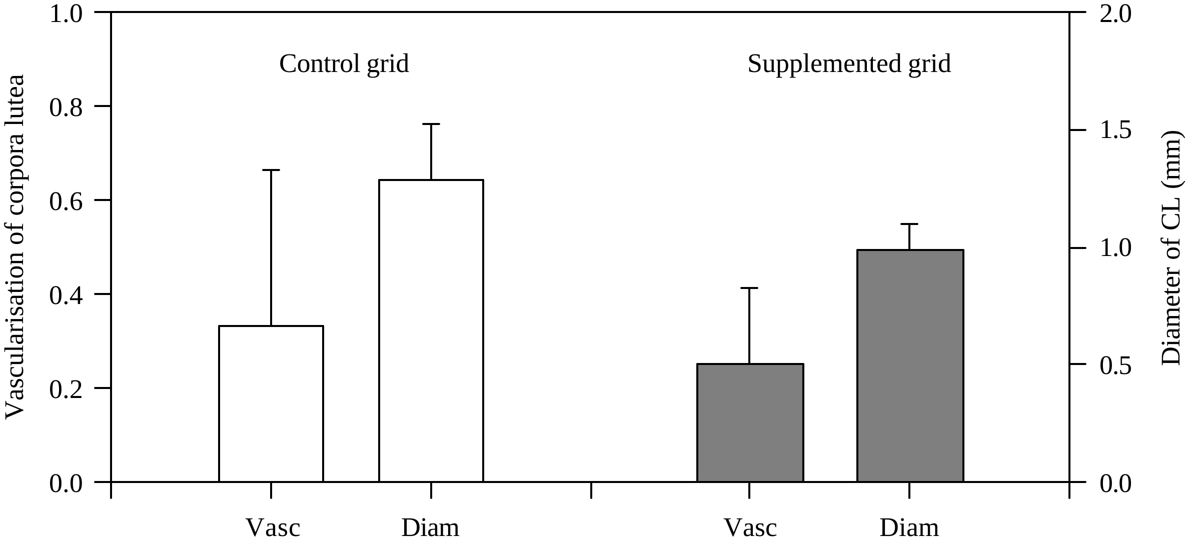

Figure 3.10: Mean vascularisation (Vasc) and diameter (Diam) of the largest corpus luteum for each female *Rhabdomys pumilio* caught after the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Values are means ? 1SEM.

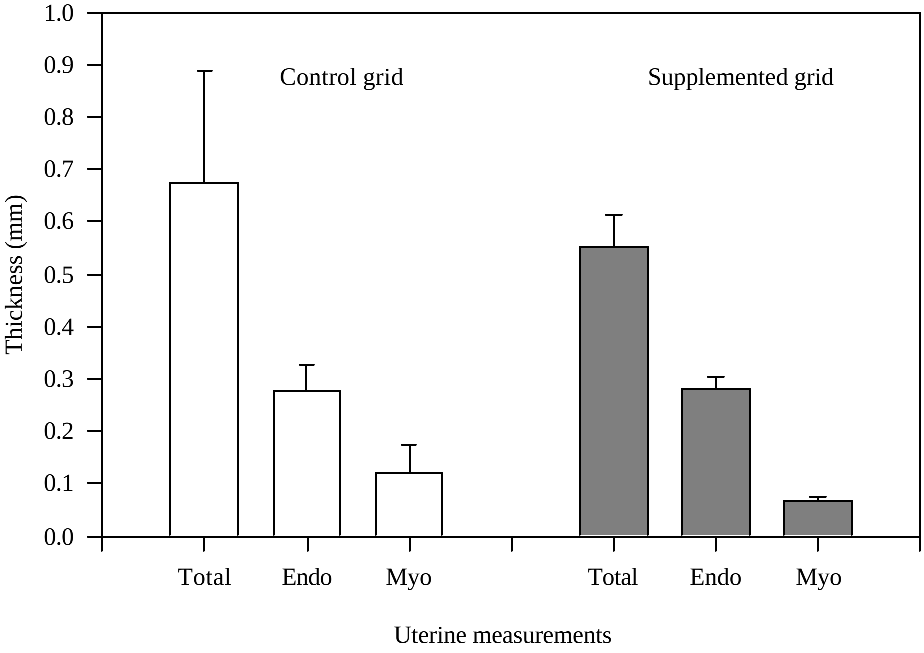

Figure 3.11: Mean thicknesses of the uterine wall (Total), the endometrium (Endo) and myometrium (Myo) of non-pregnant female *Rhabdomys pumilio* caught after the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Values are means ? 1SEM.

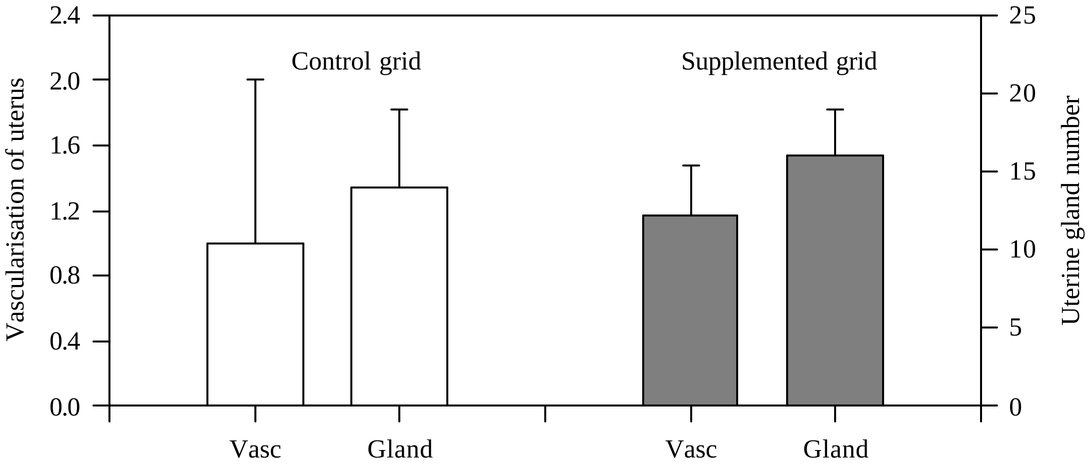

Figure 3.12: Mean vascularisation (Vasc) and number of uterine glands (gland) for the uterine walls of non-pregnant female *Rhabdomys pumilio* caught after the winter 2000 food supplementation experiment in Thomas Baines Nature Reserve. Values are means ? 1SEM.

FIELD EXPERIMENT 2001:

During the winter of 2001, a second food supplementation experiment was conducted in Thomas Baines Nature Reserve, using the same grid site as in 2000, to re-test the effects of additional food on the reproductive activity of *Rhabdomys pumilio*.

3.3) Methods:

3.3.1) *Field data:*

The methods for this field study were the same as those used in 2000 except for the following alterations. Firstly, since the single row of traps set between the two trapping grids during 2000 caught only a maximum of four marked mice per three-day trap session, it was not used in this experiment. Secondly, a rain gauge was erected within the study area to allow for the collection of rainfall data during the experiment.

3.3.2) *Sacrifice, tissue collection and blood collection:*

The methods for sacrifice, tissue and blood collection were the same as those used in the 2000 field experiment except that females which were apparently pregnant through external examination were not sacrificed, although body mass, foot length and reproductive status were recorded.

3.3.3) *Histological analysis, hormone assays, and population and statistical analyses:*

The methods of analysis were the same as those used in the first field experiment.

3.4) Results:

3.4.1) *Climate:*

Data from the South African Weather Services indicated that the winter of 2001 was slightly warmer and wetter than for previous years (see Figures 2.4 & 2.5). Climatic data collected in the field indicated that the winter daily maximum temperatures fell below 20^{o}C on only a few days in mid-June, late July and early August. The coldest day occurred in June when the maximum temperature was below 15^{o}C. The minimum temperatures ranged between 4^{o}C and 17^{o}C (Figure 3.13). The mean monthly maximum and minimum temperatures did not differ significantly ($P > 0.05$), although May was the warmest month and July the coldest month (Table 3.7).

Table 3.7: Mean monthly maximum and minimum field temperatures for the winter 2001 food supplementation experiment in Thomas Baines Nature reserve. Values are means ? 1SEM.

Month	Minimum temperature	Maximum temperature
May	11.63 ± 1.51	28.88 ± 1.01
June	9.57 ± 0.48	24.43 ± 2.73
July	7.20 ± 1.59	23.60 ± 1.89
August	7.33 ± 1.20	26.00 ± 3.51

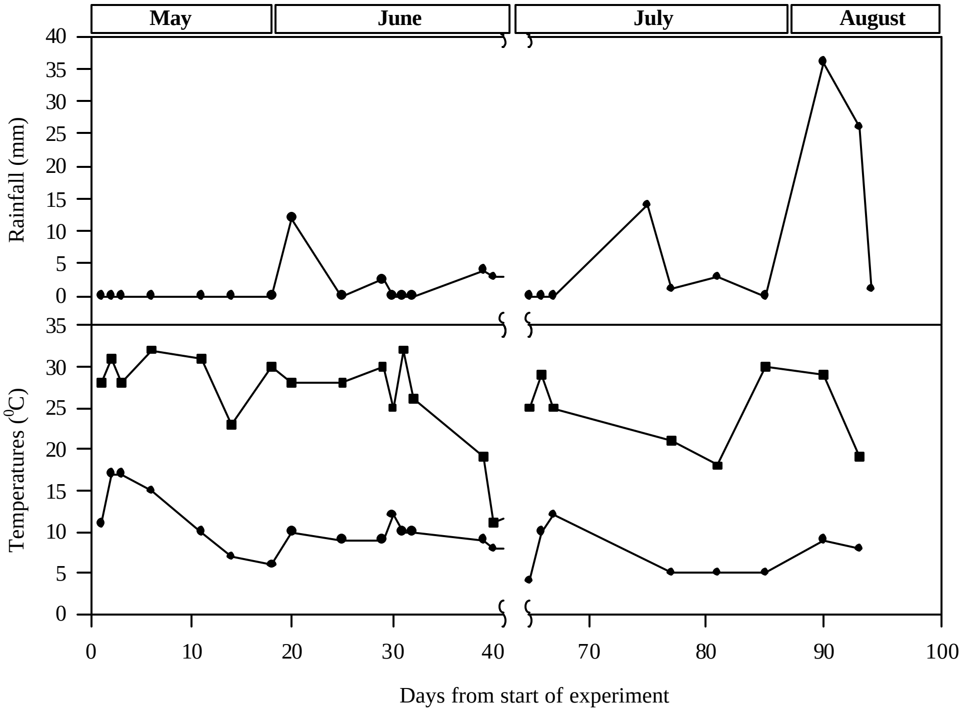

Figure 3.13: Rainfall (top) and minimum (circles) and maximum (squares) temperatures (bottom) recorded in the field at times of feeding and trapping in Thomas Baines Nature Reserve during the winter 2001 food supplementation experiment. Days 41 to 65 are omitted as data were not collected during this time.

Rainfall in the first three months of winter was low, with less than 15mm falling between measurements. However, towards the end of July and in August large amounts of rain fell over short periods of time, with the largest amount being 35mm, which fell in the last week of the experiment (Figure 3.13).

3.4.2) *Field data:*

During the experiment 80kg of food was dispensed on the supplemented grid. Faeces similar to those produced by *Rhabdomys pumilio* were observed in and around the petri dishes and it was therefore assumed that *Rhabdomys pumilio* were utilising this food source. During the experiment two mice (one from each grid) moved from one grid to the other and were considered to be immigrants and therefore left on the 'new' grid, where they remained for the rest of the experiment.

Mark-recapture analysis of the field data indicated that the population of *Rhabdomys pumilio* was significantly higher in June than in the other months on the supplemented grid, while the estimated population sizes for the control grid remained stable over the experiment (Table 3.8). Except in May, the estimated population sizes were significantly lower on the control grid than on the supplemented grid (Table 3.8).

Table 3.8: Estimated population sizes of *Rhabdomys pumilio* on two grids in the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve. Note that food supplementation began after the May trapping session and that mice were removed from the population during the August trapping session, hence estimates for this month have not been calculated. (See Table 3.9 for total number of first captures).

	Control grid	**Supplemented grid**
May	16	20
June	23	61
July	23	57
August	-	-

Log-linear analyses on the field data (Table 3.9) showed that significantly more adults were caught during the experiment than other age classes ($P < 0.001$), significantly more mice were caught on the supplemented grid than on the control grid ($P < 0.001$), and the total number of mice caught on both grids was significantly higher in July than in the other months ($P = 0.023$; Tables 3.9, 3.10). A large number of juveniles ($n = 17$) were caught on the supplemented grid in June, while in May and August no juveniles were caught on the supplemented grid. On the control grid, no juveniles were caught during August. Significantly more reproductively active mice were caught on the supplemented grid ($P < 0.001$) and there was a significant increase in the number of reproductively active mice caught, from May to August, on both grids ($P = 0.027$; Tables 3.9, 3.10).

Table 3.9: Population dynamics of *Rhabdomys pumilio* trapped on the control and supplemented grids during the winter 2001 food supplementation in Thomas Baines Nature Reserve. Numbers are of individual mice, excluding recaptures.

	Control Grid				Supplemented Grid			
	May	June	July	August	May	June	July	August
Number of mice caught:								
Juveniles	6 (6?)	4 (2? ; 2?)	5 (5?)	0	0	17 (8? ; 9?)	7 (4? ; 3?)	0
Sub-adults	4 (2? ; 2?)	5 (2? ; 3?)	7 (3? ; 4?)	4 (4?)	7 (4? ; 3?)	8 (5? ; 3?)	17 (7? ; 10?)	2 (1? ; 1?)
Adults	6 (2? ; 4?)	9 (5? ; 4?)	8 (3? ; 5?)	18 (9? ; 9?)	20 (7? ; 13?)	24 (4? ; 20?)	29 (12? ; 17?)	29 (12? ; 17?)
Total caught	16	18	20	22	27	49	53	31
% mice per trap session:								
Juveniles	38	22	25	0	0	35	13	0
Sub-adults	24	28	35	18	26	16	32	6
Adults	38	50	40	82	74	49	55	94
% mice reproductively active per age group per trap session:								
Juveniles	0	0	0	0	0	0	0	0
Sub-adults	25	20	29	0	14	13	6	0
Adults	67	100	50	72	75	79	45	93
Total N^{o} reproductively active	5	10	6	13	16	20	14	27

Table 3.10: Results of log-linear analysis of frequency tests conducted on the population data collected during the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve.

	Comparison	**P value**
No. mice caught	Months	P = 0.023
	Ages	P < 0.001
	Grids	P < 0.001
No. reproductively active	Months	P = 0.027
	Grids	P < 0.001

MANOVAs conducted on the effects of grid and month on the body mass and hind foot length of each age group (Figure 3.14), indicated that there was a significant effect of month on the mean body mass of the juveniles (P = 0.036) and on the mean hind foot length of the sub-adults (P = 0.033). Juveniles were significantly lighter in July than in June on the supplemented grid, while sub-adults on the control grid had significantly larger hind feet in July than in the other months. There was a significant effect of grid on the mean body mass of the adults (P = 0.018), where adults on the control grid were significantly lighter in August than the adults on the supplemented grid. There was a significant interaction of grid and month on the mean body mass of the sub-adults, where sub-adults caught on the control grid in May were significantly heavier than all other sub-adults caught during the experiment (Figure 3.14; Table 3.11).

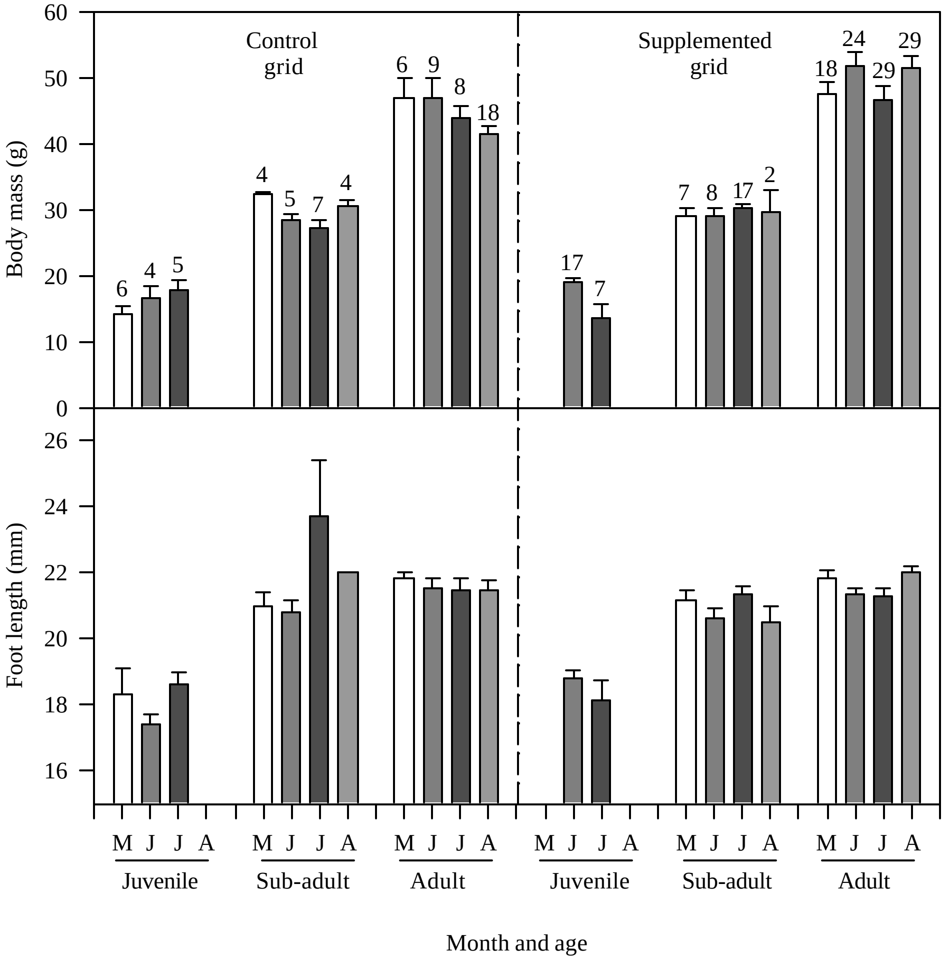

Figure 3.14: Mean body mass and hind foot length of mice trapped during the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve. Mice are classified according to their age group and the grid on which they were caught, and according to the month in which they were trapped. M, J, J, and A represent the months May, June, July and August respectively. Means are ? 1SEM. The numbers above the bars represent the sample size per group.

Table 3.11: Results of MANOVAs conducted on the mean body mass and mean hind foot length of mice from two grids for the various months in which mice were caught, during the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve.

Age group	Month	Grid	Month x Grid
Body mass			
Juvenile	P = 0.036	P = 0.634	No interaction
Sub-adult	P = 0.317	P = 0.798	P = 0.041
Adult	P = 0.467	P = 0.018	No interaction
Hind foot length			
Juvenile	P = 0.897	P = 0.284	No interaction
Sub-adult	P = 0.033	P = 0.099	No interaction
Adult	P = 0.329	P = 0.730	No interaction

Division of the mice into the nine weight classes for each month during which they were caught, indicated that in May, the mice from the control grid ranged in weight from 10.0g to 69.9g. However, 11 of these mice occurred in the lower categories (10.0–39.9g) and the lowest category (10.0-19.9g) comprised females only. Mice from the supplemented grid ranged in weight from 20.0g to 69.9g. Females dominated the weight classes 30.0-39.9g and 50.0-59.9g, while males dominated the 20.0-29.9g and 40.0-49.9g weight classes (Figures 3.15, 3.16). In June, mice on the control grid were evenly distributed through the weight classes with at least one mouse and no more than four mice occurring in each of the categories occupied (10.0-69.9g). Males and females were similarly distributed within the occupied weight classes, although no males weighed more than 59.9g and no females weighed between 50.0-59.9g. Mice from the supplemented grid ranged in weight from 10.0g to 79.9g, with two peaks occurring in the 20.0-29.9g and 50.0-59.9g categories. The former category was dominated by males, while females dominated the latter category. Females also dominated the 10.0-19g and the 30.0-39g categories, and the two highest categories occupied (60.0-79.9g; Figures 3.15, 3.16). In July, the mice from the control grid weighed less than 60.0g and were evenly distributed through the occupied classes (10.0-59.9g) with a peak in the 20.0-29.9g weight class, which was dominated by males. Mice from the supplemented grid occurred in the weight

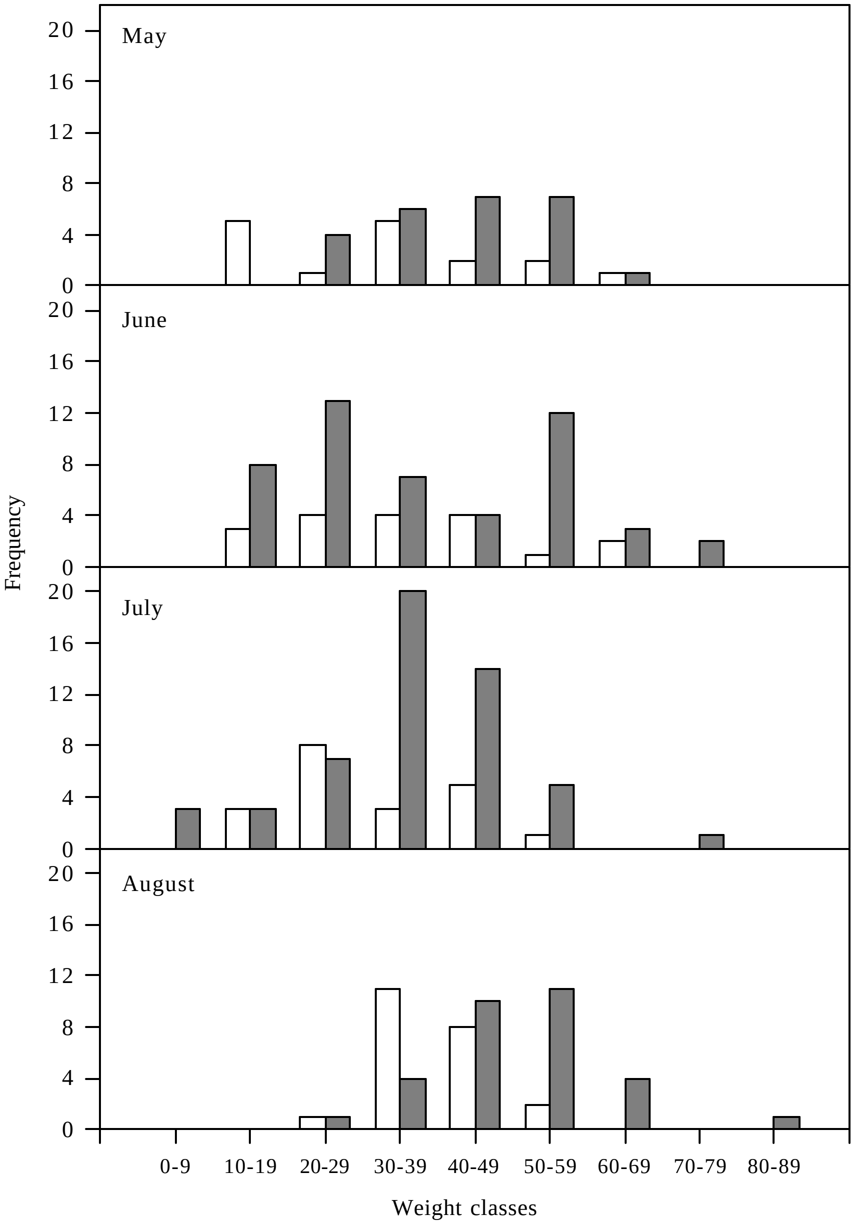

Figure 3.15: Size distribution of *Rhabdomys pumilio* from the control and experimental grids of the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve. Weight classes are in grams and tick labels have been rounded to save space (0-9g = 0.0-9.9g). Clear bars denote the control grid; shaded bars denote the supplemented grid.

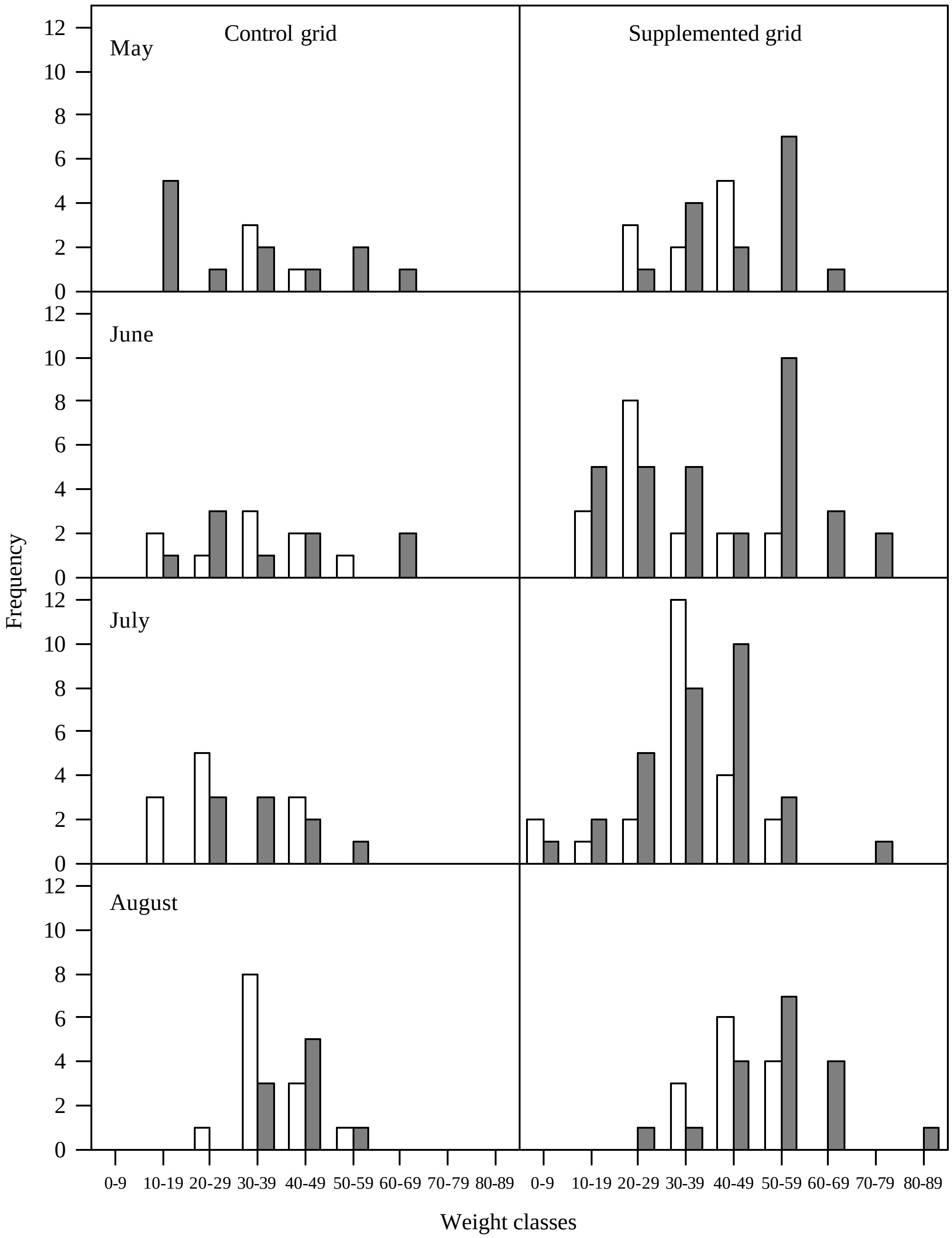

Figure 3.16: Size distribution of *Rhabdomys pumilio* from the control and experimental grids of the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve. Weight classes are in grams and tick labels have been rounded to save space (0-9g = 0.0-9.9g). Clear bars denote males; shaded bars denote females.

categories from 0.0g to 79.9g, with large numbers of mice occurring in the 30.0-39.9g weight class (n = 20, male dominated) and the 40.0-49.9g weight class (n = 14; female dominated; Figures 3.15, 3.16). In August, mice from the control grid weighed between 20.0g and 59.9g, peaking in the 30.0-49.9g categories, while mice from the supplemented grid weighed between 20.0g and 89.9g, with 21 mice occurring in the 40.0-59.9g categories. The heaviest mouse caught in August was a female from the supplemented grid, which weighed 85g (Figures 3.15, 3.16).

A MANOVA conducted on the mean body masses of the mice (Table 3.12) from the two grids for each of the months, indicated that mice on the supplemented grid were significantly heavier than those on the control grid (P = 0.006), females were significantly heavier than males (P = 0.002), and mice caught in August were significantly heavier than those caught in the first three months of the experiment (P < 0.001). However, there were no significant interactions between these factors (P > 0.05).

Table 3.12: The mean body masses of the males and females and the overall mean body mass for the mice on the control and experimental grids during the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve. Values are means ? 1SEM. (See Table 3.9 for sample sizes).

	Means	**May**	**June**	**July**	**August**
Control grid	Total	29.9 ? 3.95	34.9 ? 3.39	31.5 ? 2.58	39.4 ? 1.37
	Female	27.6 ? 5.38	36.9 ? 5.47	36.8 ? 3.29	42.0 ? 1.75
	Male	35.5 ? 2.10	33.0 ? 4.26	27.2 ? 3.44	37.5 ? 1.88
Supplemented grid	Total	42.0 ? 2.73	36.7 ? 2.45	35.9 ? 1.80	50.0 ? 2.10
	Female	47.0 ? 3.10	41.1 ? 3.20	37.3 ? 2.55	53.6 ? 3.00
	Male	33.4 ? 3.37	28.2 ? 2.82	34.1 ? 2.51	44.9 ? 2.20

3.4.3) *Dissection data:*

At the end of the experiment, a total of 35 adults (10 males and eight females from the supplemented grid and nine males and eight females from the control grid) were caught and brought back to the laboratory for dissection. Of the females, nine (eight from the supplemented grid) were visibly pregnant and were not dissected. However, on dissection of the remaining females, it was evident that all were pregnant. On the control grid, four of the males were non-scrotal, four were partly-scrotal and one was scrotal, while one of the males from the supplemented grid was non-scrotal, five were partly-scrotal and four were scrotal. At dissection there was no significant difference in the mean body masses between the males from the two grids ($P = 0.14$), however the females from the supplemented grid were significantly heavier than the females from the control grid ($P = 0.01$; Figure 3.17A). There was no significant difference in the mean hind foot lengths of the mice from either grid ($P > 0.05$; Figure 3.17B) and the mice from the two grids had similar body fat scores ($P > 0.05$), varying in score from 0 to 2 (Figure 3.17C).

The mean masses of the reproductive organs of the males were not significantly different across the grids, although in all cases (testes, epididymides and accessory glands) the males from the supplemented grid had the heavier reproductive organs (Figures 3.17D & E). Comparisons between females could not be made as no females from the supplemented grid were dissected, as they were all visibly pregnant, however data for the females from the supplemented grid have been included (Figures 3.17D & E).

3.4.4) *Histology:*

All the males were spermatogenically active with more than 80% of the seminiferous tubules containing spermatozoa, while mean sperm storage in the epididymides ranged from 56% (control grid) to 80% (supplemented grid), and the mean activity of the accessory glands was above 95% on both grids. There was no significant difference in the activity of the reproductive organs for males from the control and supplemented grids ($P > 0.1$), although the males from the control grid had the lower levels of activity (Figure 3.18).

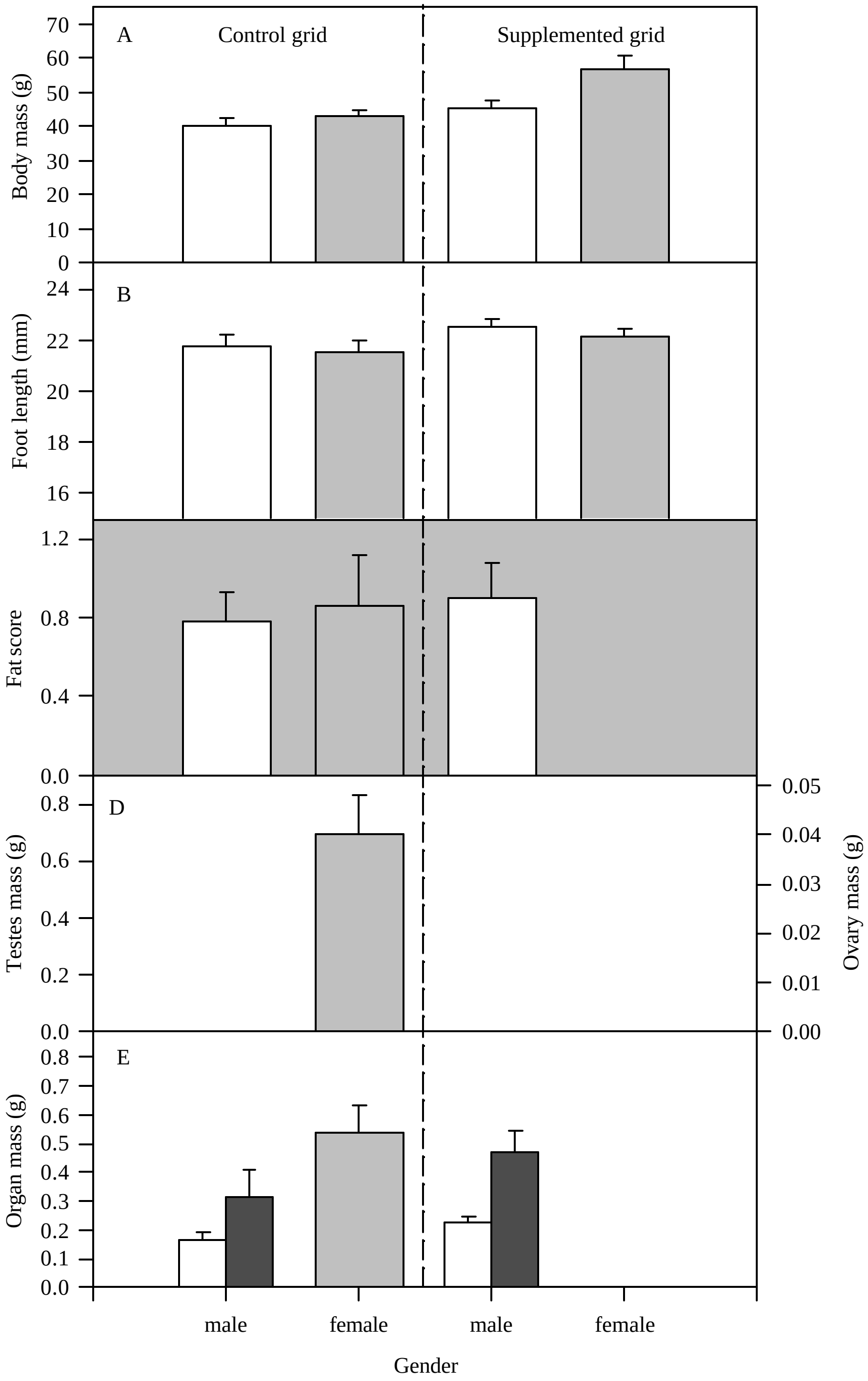

Figure 3.17: Dissection data for mice collected after the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve, indicating (A) mean body mass; (B) mean hind foot length; (C) mean fat score; (D) mean mass of the testes and ovaries; and (E) the mean mass of the epididymides (clear bars), accessory glands (cross-hatched bars) and uterine horns (dark bars). Means are ? 1SEM.

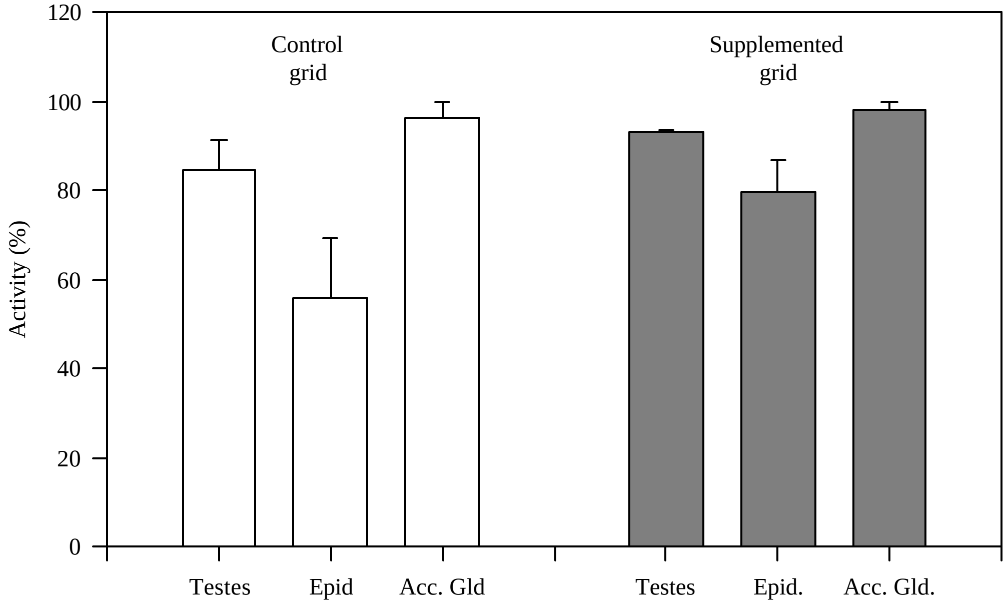

Figure 3.18: Mean activity (%) of the reproductive organs collected from male *Rhabdomys pumilio* after the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve. Epid. = epididymides; Acc. Gld. = accessory glands. Means are ? 1SEM.

Analysis of the dissected females from the control grid indicated that these females had an average of 11 secondary follicles, four Graafian follicles and four corpora lutea in their ovaries (Figure 3.19A). The corpora lutea were highly vascularised with a mean diameter of 0.97 ? 0.1mm (Figure 3.19B). The mean thicknesses of the uterine wall, endometrium and myometrium were 0.37 ? 0.07mm, 0.19 ? 0.04mm and 0.07 ? 0.01mm, respectively (Figure 3.19C). The uterine walls were well vascularised (? = 1.86 ? 0.4) with a large number of glands (? = 14.57 ? 5.06; Figure 3.19D).

3.4.5) *Hormone assays:*

Mean plasma testosterone levels of the males did not differ significantly between the two grids (P = 0.17; supplemented grid, ? = 92.3 ? 32.5ng/dL; control grid, ? = 60.3 ? 21.7ng/dL).

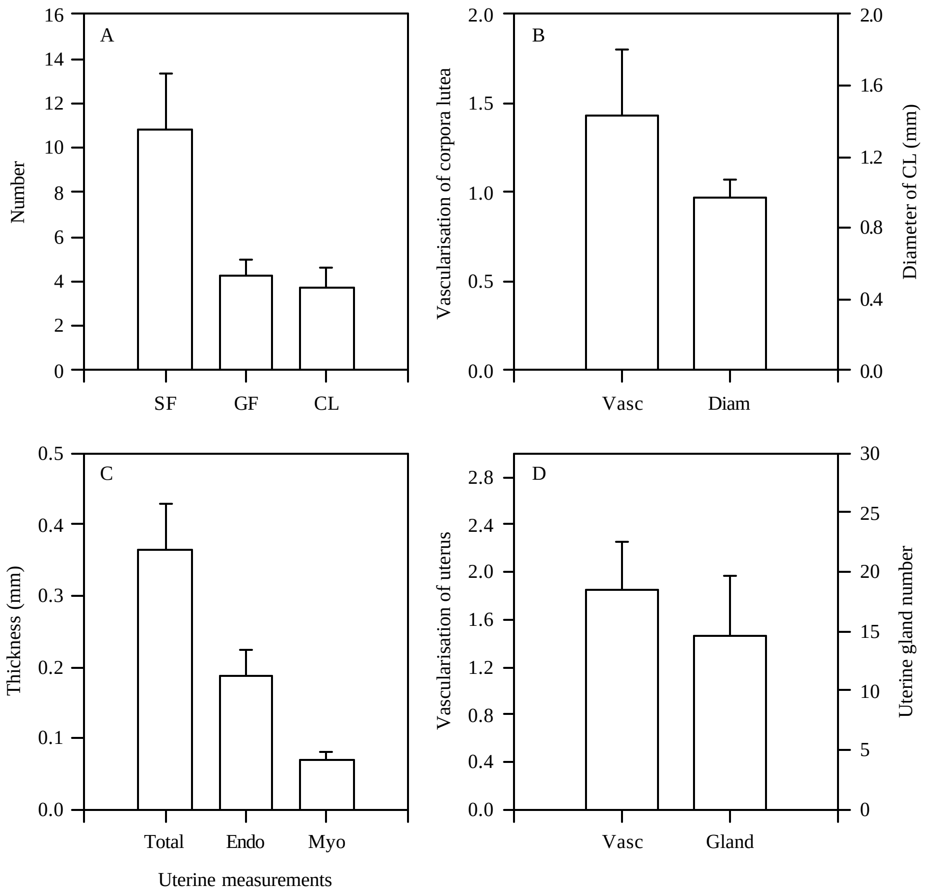

Figure 3.19: (A) Mean number of secondary follicles (SF), Graafian follicles (GF) and corpora lutea (CL) from one ovary per non-pregnant female; (B) the mean vascularisation (Vasc) and diameter (Diam) of the largest corpus luteum per ovary; (C) mean measurements of the uterine wall (Total), endometrium (Endo) and myometrium (Myo); and (D) mean vascularisation (Vasc) and number of uterine glands (Gland) for the uterine walls, analysed for non-pregnant female *Rhabdomys pumilio* caught on the control grid after the winter 2001 food supplementation experiment in Thomas Baines Nature Reserve. Means are ? 1SEM.

FOOD SUPPLEMENTATION EXPERIMENT: MOUNTAIN ZEBRA NATIONAL PARK

Because the results from the field experiments in Thomas Baines Nature Reserve showed that *Rhabdomys pumilio* remained reproductively active during the winter when conditions were mild, it was decided to conduct another food supplementation experiment in an area of the Eastern Cape which experiences harsher and more predictable winters than Grahamstown.

STUDY SITE:

The study site for the winter 2002 food supplementation experiment was located in Mountain Zebra National Park (MZNP) near Cradock. MZNP is an established nature reserve that has recently (2000) been expanded to include old farmlands (the lands have been free of livestock since late 1999). It was on one of these old farms that the study site for the food supplementation experiment was located. While the reserve is home to a variety of mammal and bird species, the study site was mainly undisturbed by game, although kudu (*Tragelaphus strepsiceros*), vervet monkey (*Cercopithecus pygerythrus*), baboon (*Papio ursinus*) and caracal (*Felis caracal*) were seen in the area during the experimental period. Signs of black rhinoceros (*Diceros bicornis*) and buffalo (*Syncerus caffer*) were also observed during the experimental period, however these were more recent disturbances as these species were only released into the old farm lands during March 2002 (buffalo) and May 2002 (black rhinoceros). The smaller mammals evident within the study site included porcupine (*Hystrix africaeaustralis*), pygmy mouse (*Mus minutoides*), four striped field mouse (*Rhabdomys pumilio*), pouched mouse (*Saccostomus campestris*), multimammate mouse (*Praomys natalensis*) and an unidentified shrew species.

Within the old farmlands, two adjacent trapping sites that were similar in slope and vegetation type were established. These sites were 400m apart and were separated by a section of thick bush. This region is classified as Karroid Merxmuellera Mountain veld type by Acocks (1975), while Low and Rebelo (1996) classify it as South Eastern mountain grassland. Both sites were situated close to a riverbed and could be divided into two levels. The upper regions being dominated by grasslands with some low bush, while the lower regions, which were closer to the river, were dominated by riverine bush (Figures 3.20, 3.21). The dominant vegetation included *Acacia karroo*, *Rhus lancea*, *Rhus longispina*, *Lycium* spp. and *Diospyros* spp. and grass species. In both sites, the trap lines were set

Figure 3.20: Representative photographs of the control grid used in the winter 2002 food supplementation experiment in MZNP. Top and middle photographs are of the upper region of the grid, the bottom photograph is of the lower region near the riverbed.

Figure 3.21: Representative photographs of the supplemented grid used in the winter 2002 food supplementation experiment in MZNP. Top and middle photographs are of the upper region of the grid, the bottom photograph is of the lower region near the riverbed.

perpendicular to the river, with five rows of ten traps each (n= 50), each trap being 10m apart.

PRE-EXPERIMENT STUDIES

Prior to the experiment, three trapping sessions were carried out (June 2001, September 2001 and March 2002) on what became the supplementation grid and one trapping session was carried out on what became the control grid (March 2002). These sessions lasted between three and six days and were part of another project. The results of these preliminary trapping sessions showed that estimated populations of *Rhabdomys pumilio* were large (>50 for all sessions on both grids; Table 3.13) and that the percentage of adult mice that were reproductively active was low in winter (June; Table 3.13). These factors indicated that MZNP had a viable population of mice capable of surviving winter in large numbers and that there was an inhibition of reproduction in winter, therefore making MZNP suitable for a food supplementation experiment.

Table 3.13: Estimated population sizes of *Rhabdomys pumilio* and percentage of reproductively active mice for two separate grids during pre-experiment studies in MZNP. Population sizes were estimated using the modified Lincoln Index and the total numbers of first time captures have been included in parentheses.

Grid	Trap session	Estimated population size	% reproductively active adults
Future supplemented grid	June 2001	89 (68)	10
	September 2001	126 (53)	20
	March 2002	110 (76)	61
Future control grid	March 2002	56 (43)	52

FIELD EXPERIMENT 2002:

3.5) Methods:

3.5.1) *Field data collection:*

Although the methods for this supplementation experiment were similar to those used in the Thomas Baines Nature Reserve experiments, some alterations were made. Firstly trapping sessions were based on the availability of accommodation at MZNP and my ability to be out of Grahamstown for four consecutive days. Trapping therefore occurred from 9-12 May, 24-27 June, 25-28 July and 8-10 August. The last trapping session was conducted three months after the start of the experiment. Trap sessions lasted three nights and traps were checked twice a day starting on the afternoon of day one until the morning of day four, after which traps were packed away until the next trapping session. Thus traps were checked six times, as in the previous experiments in Thomas Baines Nature Reserve.

The methods for food supplementation were altered to allow for extended feeding without the need to replenish the food every second day. Rabbit pellets (16% protein, 17% fibre, energy content of 17.05kJ/g; Epol, Johannesburg, RSA), on which *Rhabdomys pumilio* can survive and breed in the laboratory (pers. obs.), were used as the food supplement. Two-litre plastic bottles were filled with food and then inverted. A flap was cut out of the bottle to allow food to be pulled out by rodents. The bottle was then secured with cable ties to a metal stake hammered into the ground. This device was tested for a week at MZNP (March 2002), and this indicated that mice were able to gain access to the food, that the dispenser was not attractive to vervet monkeys and that the dispenser was waterproof. Two of the feeders were filled with colour-dyed (food colouring) food to test whether *Rhabdomys pumilio* were likely to use the feeders. Two *Rhabdomys pumilio* were caught in the traps near these feeders and, on immediate dissection, it was evident that they had eaten food from the feeders, as food dye was present in their stomach contents. Overall this device allowed me to have supplementation food available at each trap position (50 bottles used, one bottle at each trap point; Figure 3.22) for two weeks. Thus the food dispensers were refilled with pellets every second weekend. During the last trapping session, observations were carried out in the field to determine if *Rhabdomys pumilio* were still using the feeders.

The data that were collected for trapped *Rhabdomys pumilio* and the methods used to collect these data were similar to those used in the food supplementation experiments conducted in Thomas Baines Nature Reserve, with a few changes. Foot length data were not collect during May 2002, however foot length was measured for the other trap sessions. Mean monthly climate data (minimum and maximum temperatures and mean rainfall) for the experimental period were obtained from the South African Weather Services for the Cradock region, and mean monthly rainfall data were obtained from the weather station at MZNP.

Figure 3.22: Feeding stations used in the winter 2002 food supplementation experiment in MZNP. Left: inverted plastic 2 L bottle with flap at base and attached to metal spike with cable ties. Top right: Feeder placed near trap. Bottom right: Food scraps near mouse burrow; food was taken from the feeder and remnants of food formed a clear trail to a hole occupied by *Rhabdomys pumilio* at the base of a tree.

3.5.2) *Sacrifice, tissue collection and blood collection:*

The methods used for sacrifice and tissue and blood collection were the same as those previously described.

3.5.3) *Histological analysis:*

In addition to the methods used in the first two experiments, a more detailed analysis of spermatogenesis was undertaken. For each male, 20 seminiferous tubules were examined and assessed for spermatozoa numbers, the number of layers of germ cells comprising the seminiferous epithelium and the thickness of the epithelial layer. The number of spermatozoa were assessed in a semi-quantitative manner according to the number of spermatozoa in the lumen of a tubule: 0 = no spermatozoa; 1 = 1 to 10 spermatozoa; 2 = 11 to 50 spermatozoa; 3 = 51 to 100 spermatozoa; and 4 = more than 100 spermatozoa. The number of layers of germ cells comprising the wall of the seminiferous tubule was assessed by finding a region of the wall of the seminiferous tubule in which all the cells were in focus and counting the number of nuclei from the outside towards the lumen. The thickness of this layer was then measured along this chosen line of cells, using an optical micrometer. These assessments were repeated 20 times per animal and mean values for these observations were then obtained for each animal.

3.5.4) *Hormone assays:*

Blood was assayed for testosterone concentrations, as previously described. Plasma leptin levels were measured using a multi-species leptin RIA kit (Linco; USA). It utilises ^{125}I-labelled Human leptin and multi-species leptin antiserum and analyses the blood using a double antibody/PEG technique. The kit can detect leptin to a level as low as 1.0ng/ml and has a 73% specificity for mouse leptin. The intra- and inter- assay variations were below 4% and 10%, respectively. Since it was not possible to validate the leptin assay kit for *Rhabdomys pumilio*, hormone levels will be referred to as levels of hormone-like immunoreactivity.

3.5.5) *Population and statistical analyses:*

Population and statistical analyses were conducted as previously described.

3.6) Results:

3.6.1) *Climate:*

The mean monthly temperatures for Cradock for 2002 were similar to the means calculated for the last 10 years (1992-2001), although the maximum temperatures for June, July and August were slightly lower than average and the mean minimum temperatures for May, July and August were slightly higher than average (see Figure 2.6). However, June was colder than average and at times the minimum daily temperature fell below $0^{o}C$ and frost occurred. Snow also fell during the third week of July. Rainfall was higher than average in June in the Cradock area and in MZNP (see Figures 2.7 & 2.8).

3.6.2) *Field data:*

During the experiment, 350kg of rabbit food was dispensed on the supplemented grid. During the experiment it was noted that runways belonging to mice were lined with shredded rabbit pellets and similar evidence of rabbit pellet consumption was noted at the entrance to mouse holes (see Figure 3.22). During the last trapping session, two independent observations were made of *Rhabdomys pumilio* utilising the feeders and it was assumed that they had done so throughout the experiment.

The estimated population sizes on the supplemented grid ranged between 74 and 91 individuals (May to July), while on the control grid it ranged between 60 and 74 individuals (May to July; Table 3.14). The estimated population sizes were significantly greater on the supplemented grid ($P = 0.04$), however there was no significant change in the estimated sizes from May (pre-supplementation) to July (animals were removed from the study area in August and thus estimated population sizes for the two grids were low).

Table 3.14: Estimated population sizes of *Rhabdomys pumilio* on two grids in the winter 2002 field supplementation experiment in MZNP. Note that supplementation began after the May trapping session and that mice were removed during the August trapping session, hence estimates for this month have not been calculated. (See Table 3.15 for total number of first captures).

	Control grid	**Supplemented grid**
May	74	91
June	71	84
July	60	74
August	-	-

May was the only month during which juvenile *Rhabdomys pumilio* were caught, and the numbers were similar on the two grids (Table 3.15). Sub-adults and adults were trapped throughout the experimental period, sub-adults occurring in larger numbers at the start of the winter period (May and June) and adults occurring in similar numbers throughout (Table 3.15). Significantly more adults were caught during the experiment than other age classes ($P < 0.01$), and significantly more mice of all ages were captured during May ($P = 0.02$), however there was no significant difference in the number of captures between the two grids (Table 3.16). Significantly more reproductively active mice were caught during May ($P = 0.03$) than the other months and throughout the experiment, the supplemented grid had significantly higher numbers of reproductively active mice than the control grid ($P < 0.01$; Tables 3.15, 3.16).

MANOVAs conducted to test the effects of grid and month on body mass and hind foot length of sub-adults and adults (Figure 3.23) indicated significant grid effects on adult mean body mass and hind foot length. The adults from the supplemented grid had the higher mean body mass and mean hind foot lengths compared to the adults on the control grid ($P < 0.01$; Table 3.17). Month had a significant effect on both sub-adult and adult foot lengths, where sub-adults had significantly larger feet in June compared to the other months and adults had significantly larger feet in June and July compared to August ($P < 0.01$; Table 3.17). There were no significant interactions between grid and month ($P > 0.05$).

Table 3.15: Population dynamics of *Rhabdomys pumilio* trapped on the control and supplemented grids in MZNP during the winter 2002 food supplementation experiment. Numbers are of individual mice, excluding recaptures.

	Control Grid				Supplemented Grid			
	May	June	July	August	May	June	July	August
Number of mice caught:								
Juveniles	6 (1? ; 5?)	0	0	0	7 (1? ; 6?)	0	0	0
Sub-adults	15 (6? ; 9?)	16 (5? ; 11?)	11 (1? ; 10?)	12 (2? ; 10?)	23 (14? ; 9?)	9 (4? ; 5?)	5 (1? ; 4?)	7 (2? ; 5?)
Adults	24 (14? ; 10?)	19 (13? ; 6?)	24 (18? ; 6?)	27 (16? ; 11?)	30 (15? ; 15?)	30 (17? ; 13?)	28 (16? ; 12?)	27 (15? ; 12?)
Total caught	45	35	35	39	60	39	33	34
% mice per trap session:								
Juveniles	13	0	0	0	12	0	0	0
Sub-adults	33	46	31	31	38	23	15	21
Adults	54	54	69	69	50	77	85	79
% mice reproductively active per age group per trap session:								
Juveniles	0	0	0	0	0	0	0	0
Sub-adults	7	0	0	0	26	0	0	14
Adults	46	21	38	37	43	30	68	55
Total N^{o} reproductively active	12	4	9	10	19	9	19	16

Table 3.16: Results of Log-linear analysis of frequency tests conducted on the population field data collected during the winter 2002 food supplementation experiment in MZNP.

	Comparison	**P value**
No. mice caught	Months	P = 0.021
	Ages	P < 0.001
	Grids	P = 0.510
No. reproductively active	Months	P = 0.034
	Grids	P = 0.004

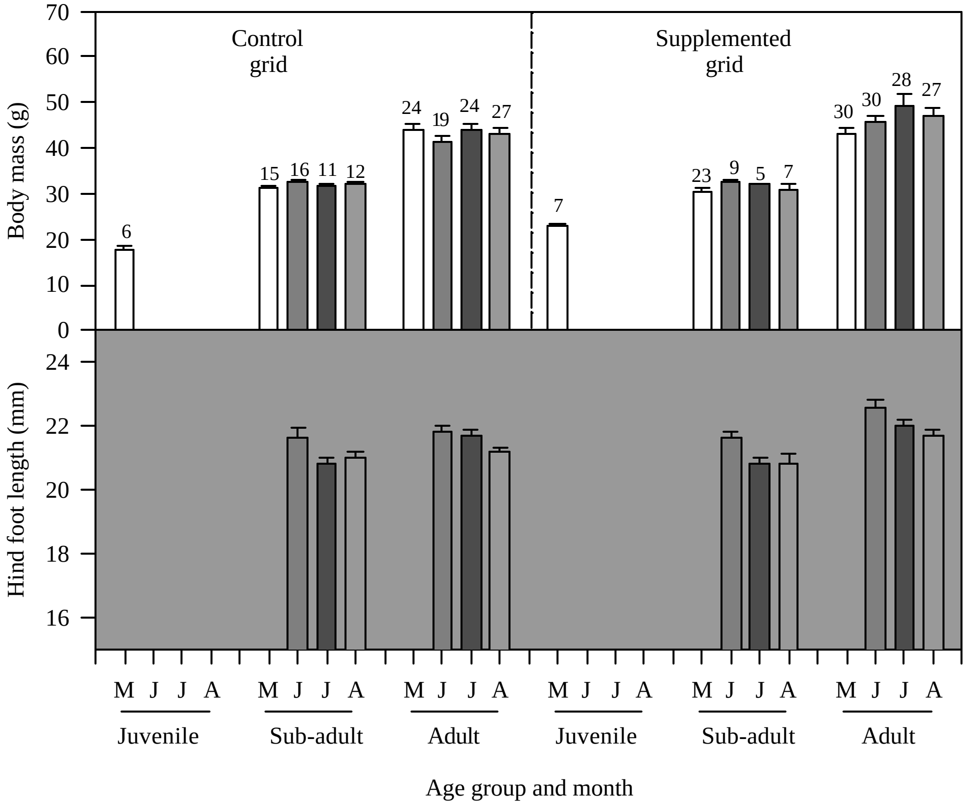

Figure 3.23: Mean (? 1SEM) body mass and hind foot length for mice trapped during the winter 2002 food supplementation experiment in MZNP. Mice are classified according to their age and the grid on which they were caught, and according to the month in which they were trapped. M, J, J and A represent the months May, June, July and August respectively. Numbers above the bars represent the sample size per group.

Table 3.17: Results of MANOVAs conducted on the mean body mass and mean hind foot length of mice from two grids for the various months in which mice were caught, during the winter 2002 food supplementation experiment in MZNP.

Age group	Month	Grid	Month x Grid
Body mass			
Sub-adult	P = 0.087	P = 0.378	P = 0.787
Adult	P = 0.154	P = 0.005	P = 0.245
Hind foot length			
Sub-adult	P = 0.007	P = 0.867	P = 0.942
Adult	P = 0.001	P = 0.001	P = 0.470

In May, the size distribution of *Rhabdomys pumilio* within the two grids was similar and the majority of mice from both grids weighed between 20.0g and 49.9g (Figure 3.24). The distribution patterns for male and females within the weight classes were similar for both grids (Figure 3.25). During June both grids were dominated by mice weighing between 30.0g and 39.9g, with this class being male dominated on the supplemented grid and female dominated on the control grid (Figures 3.24, 3.25). On both grids, in July and August the size distributions of the mice were similar, with a decrease in the number of mice from the 30.0-39.9g weight class through to the higher weight classes (Figure 3.24). For both grids, the lower weight classes were dominated by females, although in August, the largest mouse caught on the supplemented grid was a female and the lightest a male (Figure 3.25).

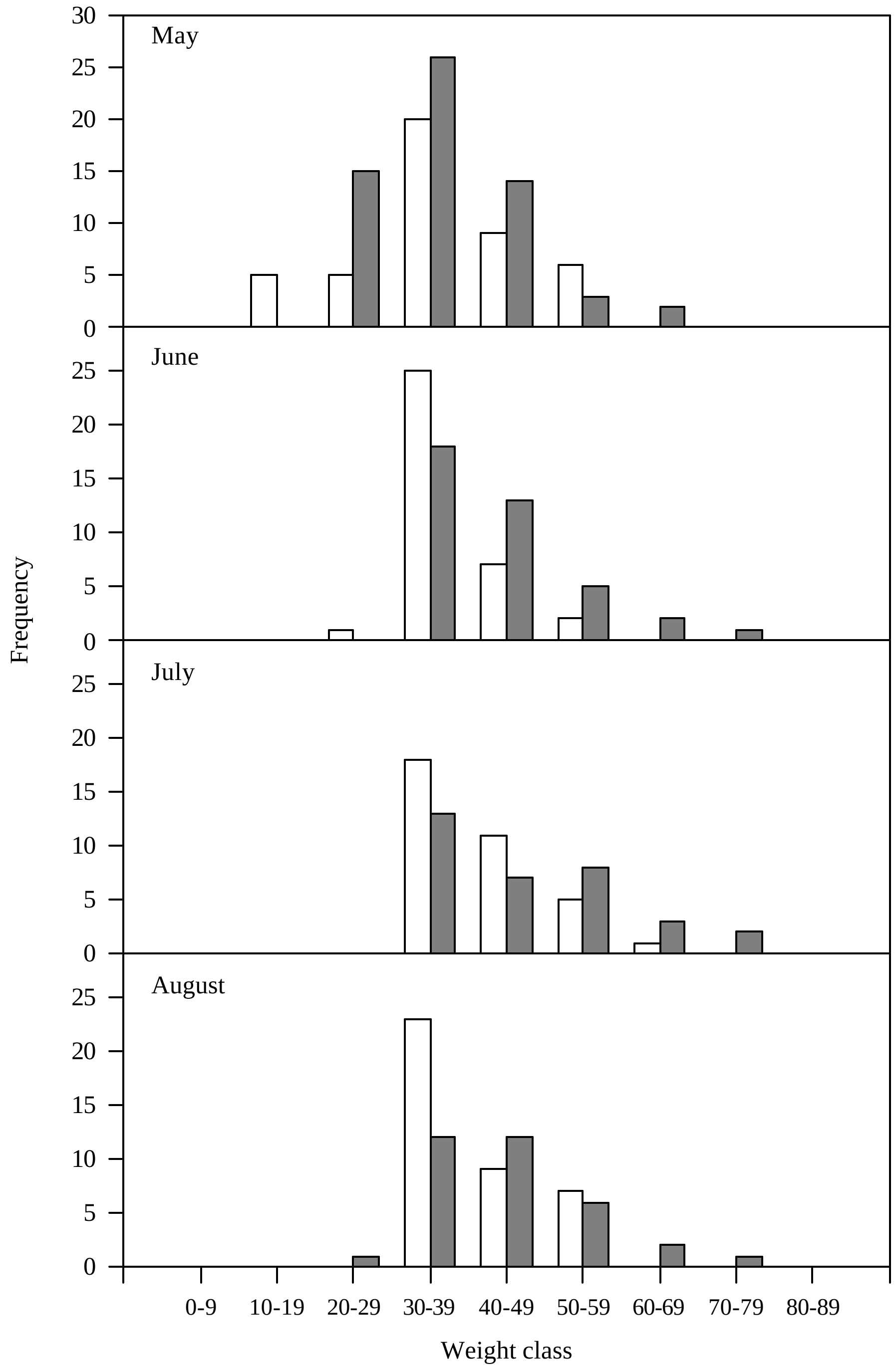

Figure 3.24: Size distribution of *Rhabdomys pumilio* from the control and experimental grids of the winter 2002 food supplementation experiment in MZNP. Weight classes are in grams and tick labels have been rounded to save space (0-9g = 0.0-9.9g). Clear bars denote the control grid; shaded bars denote the supplemented grid.

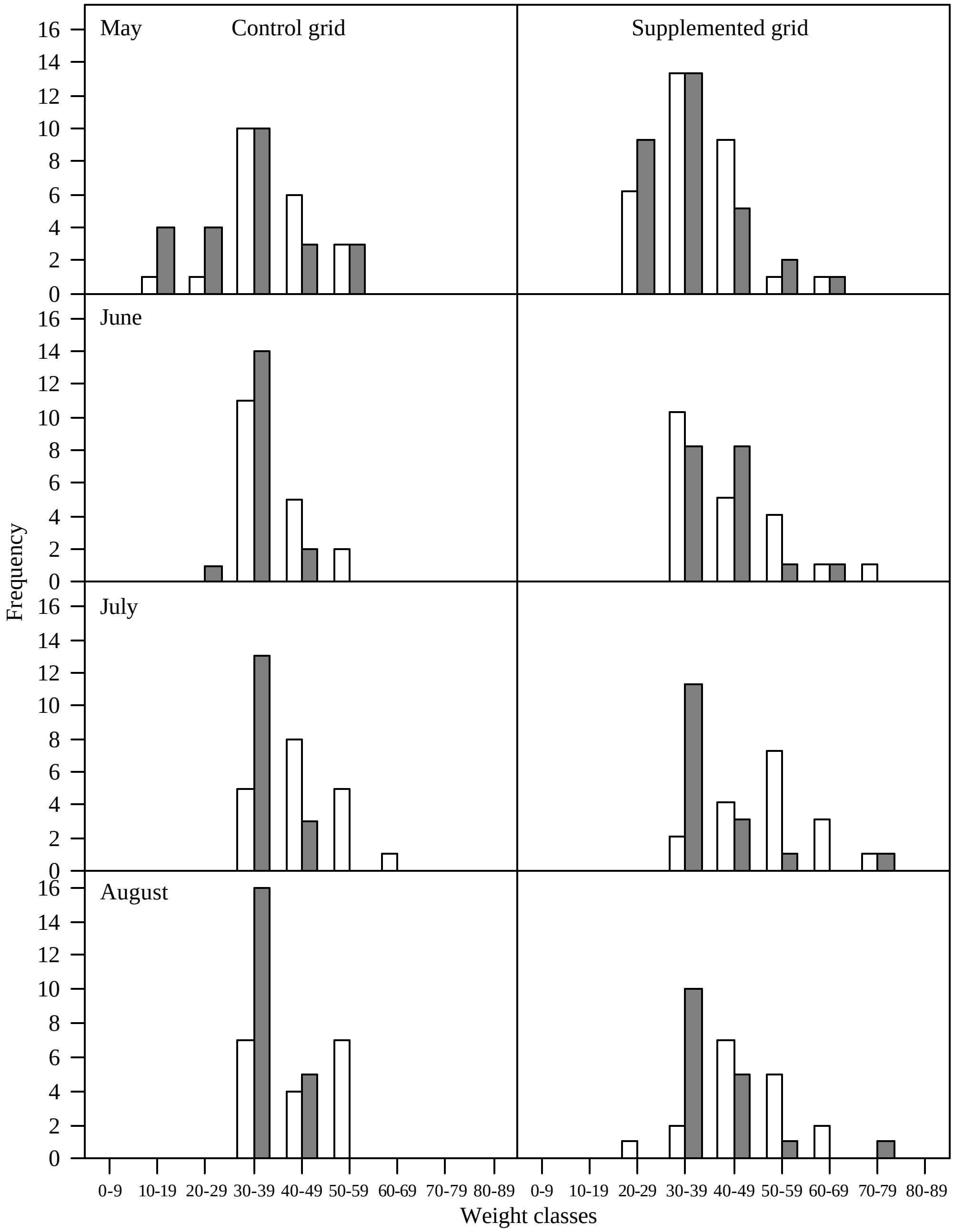

Figure 3.25: Size distribution of *Rhabdomys pumilio* from the control and experimental grids of the winter 2002 food supplementation experiment in MZNP. Weight classes are in grams and tick labels have been rounded to save space (0-9g = 0.0-9.9g). Clear bars denote males; shaded bars denote females.

A MANOVA conducted on the mean body masses of the mice from the two grids for each month (Table 3.18) indicated that males were significantly heavier than the females ($P < 0.001$), that the mice on the supplemented grid were significantly heavier than those on the control grid ($P < 0.001$) and that in July and August, mice were significantly heavier than those caught in May ($P < 0.001$). There was a significant interaction between gender and month, where males were significantly heavier than the females especially in July and August ($P = 0.01$). Although there was no significant interaction between grid and month ($P = 0.05$), it was evident that mice trapped on the two grids in May were of similar mass, however for the rest of the year, the mice from the supplemented grid were heavier than those on the control grid. There was no significant interaction between grid, month and gender ($P > 0.05$).

Table 3.18: The mean body masses of the males and females and the overall mean body mass for the mice on the control and experimental grids during the winter 2002 food supplementation experiment in MZNP. Means are ? 1SEM. (See Table 3.15 for sample sizes).

	Means	**May**	**June**	**July**	**August**
Control grid	Total	36.2 ? 1.60	37.2 ? 1.04	40.2 ? 1.35	39.7 ? 1.21
	Female	34.9 ? 2.48	34.4 ? 1.15	34.7 ? 1.14	35.7 ? 0.91
	Male	37.8 ? 1.84	39.9 ? 1.47	44.9 ? 1.68	44.4 ? 1.90
Supplemented grid	Total	36.0. ? 1.19	42.6 ? 1.56	46.8 ? 2.20	43.8 ? 1.90
	Female	35.1 ? 1.84	40.9 ? 1.92	40.4 ? 2.95	40.0 ? 2.33
	Male	36.9 ? 1.53	44.1 ? 2.38	52.8 ? 2.55	47.6 ? 2.76

3.6.3) *Dissection data:*

At the end of the experiment, equal numbers of males and females were trapped on the two grids, resulting in a total of 40 mice being caught and brought into the laboratory for dissection. All the females from the control grid were non-perforate, while five of the males from this grid were non-scrotal, three were partly-scrotal and two scrotal. All the females, except one, from the supplemented grid were non-perforate, while six of the males were scrotal and the remaining four mice partly-scrotal.

At dissection, the mean body masses of the males from the two grids were not significantly different, although males from the supplemented grid were heavier (Figure 3.26A). In contrast, the females from the supplemented grid were significantly heavier than the females from the control grid ($P = 0.03$). The hind foot lengths of all the mice were not significantly different (Figure 3.26B) and there was no significant difference in the mean fat scores of the mice from the two grids (Figure 3.26C). The mice from the supplemented grid had significantly heavier testes, ovaries, epididymides and uteri than the mice from the control grid ($P < 0.05$), however the mean mass of the accessory glands for the males was similar for both grids (Figure 3.26 D & E).

3.6.4) *Histology:*

All the males from the two grids were spermatogenically active and there was no significant difference in the mean percentages of spermatogenic activity. There was no significant difference in the amount of sperm in storage for the males from the two grids, although the mice from the control grid had lower epididymal activity than the mice from the supplemented grid ($P > 0.05$ for both; Figure 3.27). The mean activity of the accessory glands of the mice from the supplemented grid was significantly greater than that of the mice from the control grid ($P < 0.01$; Figure 3.27).

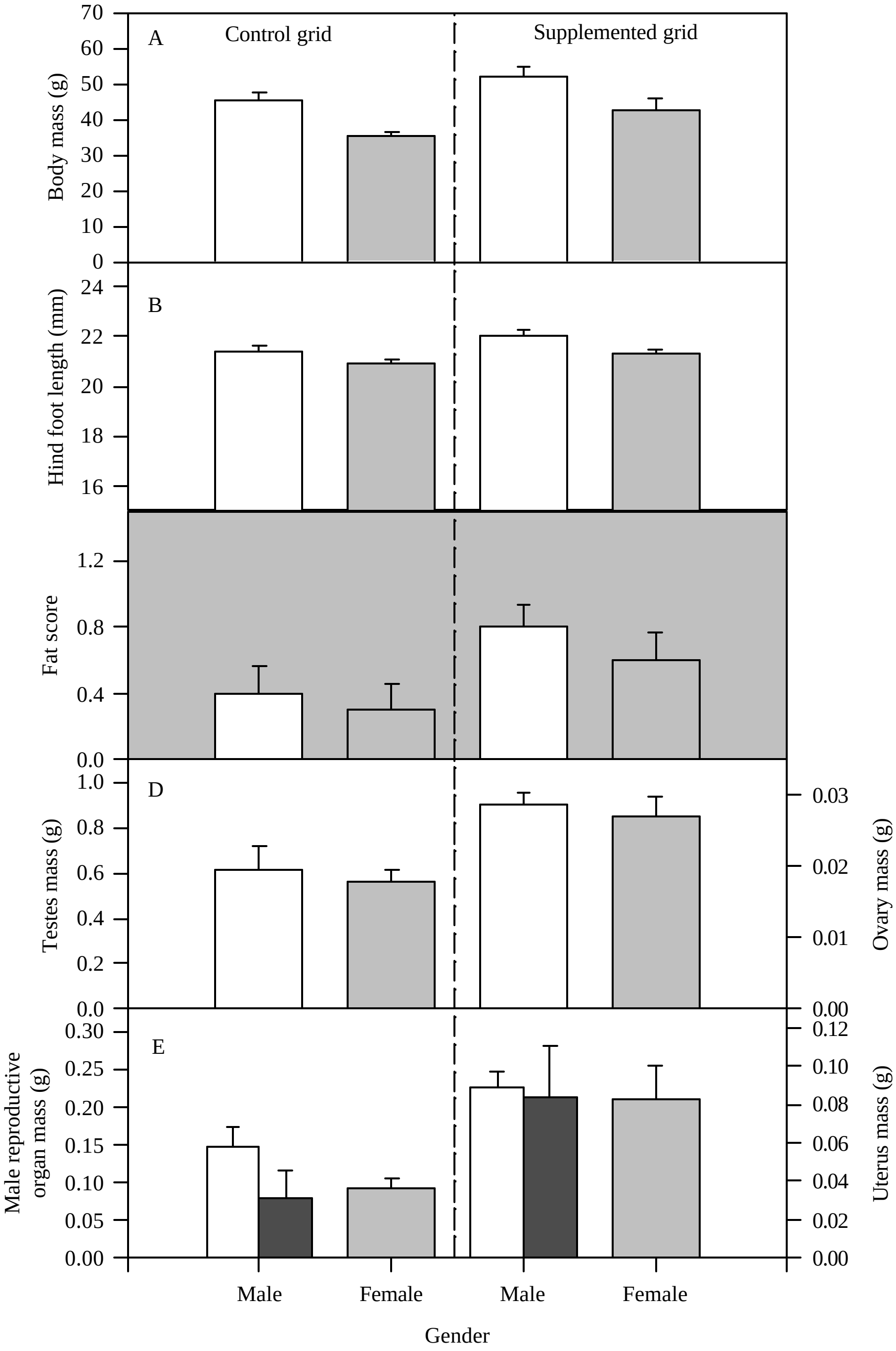

Figure 3.26: Dissection data for mice collected after the winter 2002 food supplementation experiment in MZNP, indicating (A) mean body mass; (B) mean hind foot length; (C) mean fat score; (D) mean mass of the testes and ovaries; and (E) the mean mass of the epididymides (clear bars), accessory glands (cross-hatched bars) and uterine horns (dark bars). Means are ? 1SEM.

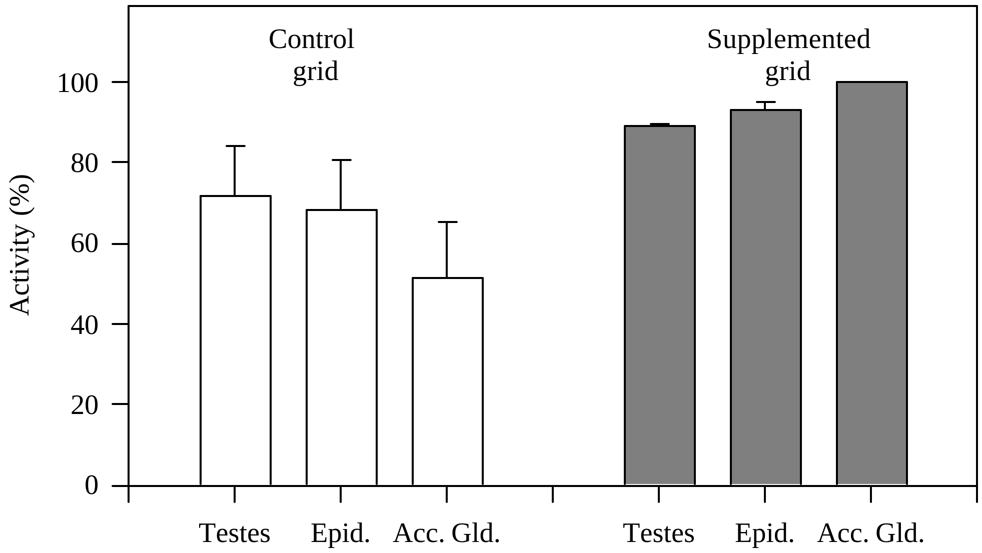

Figure 3.27: Mean activity (%) of the reproductive organs collected from male *Rhabdomys pumilio* after the winter 2002 food supplementation experiment in MZNP. Epid = epididymides; Acc. Gld. = accessory glands. Means are ? 1SEM.

Estimates of spermatozoa numbers within the seminiferous tubules for the males indicated no significant differences (P = 0.06), although the estimates were higher for the mice from the supplemented grid (Figure 3.28). There was a significant difference in the number of layers of germ cells comprising the seminiferous epithelium (P = 0.02), where the males from the supplemented grid had a greater number of layers of germ cells than the mice from the control grid had (Figure 3.28). However, the thickness of this epithelial layer did not differ significantly (P = 0.07).

Although there were differences in the number of the ovarian follicles, and the size and vascularisation of the corpora lutea within the ovaries of females from the two grids, these differences were not statistically significant (P > 0.05; Figures 3.29, 3.30).

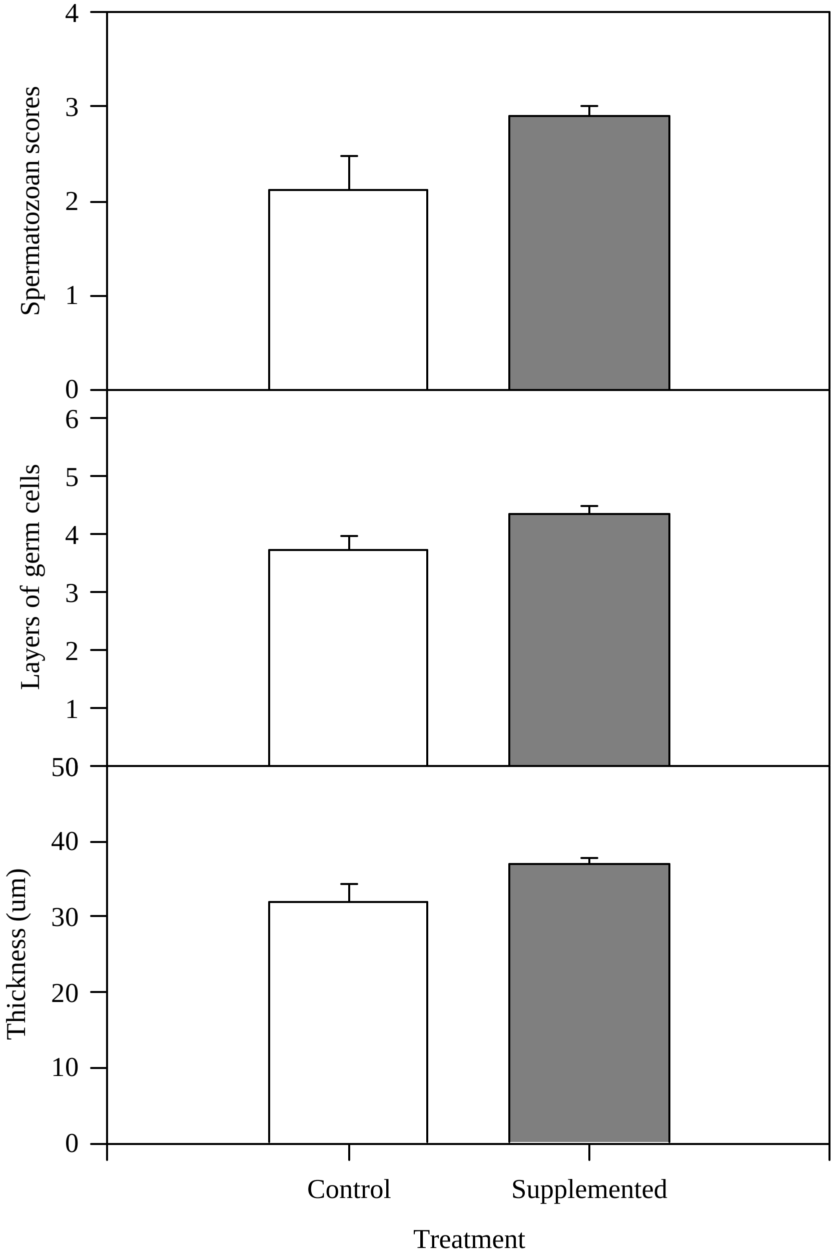

Figure 3.28: Mean (? 1SEM) score for the abundance of spermatozoa (spermatozoan score), layers of germ cells and thickness of the seminiferous epithelium of mice collected after the winter 2002 food supplementation experiment in MZNP.

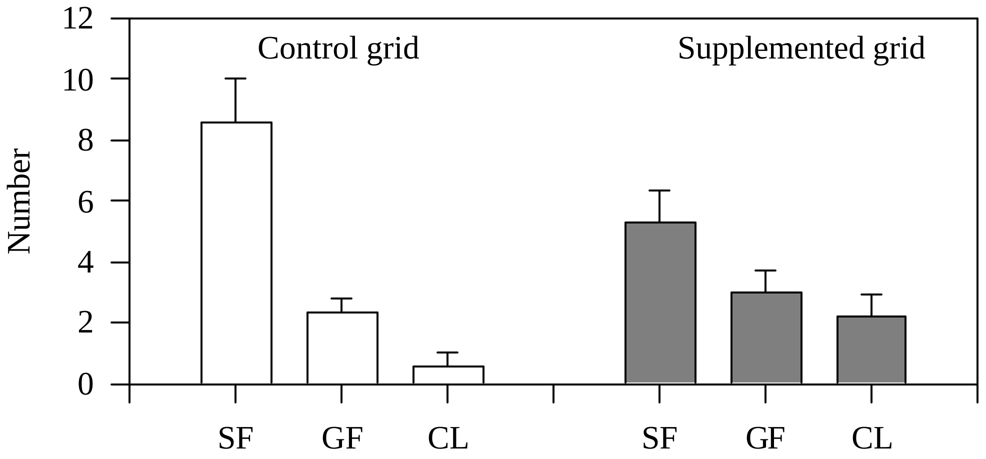

Figure 3.29: Mean number (? 1SEM) of secondary follicles (SF), Graafian follicles (GF) and corpora lutea (CL) from one ovary per female *Rhabdomys pumilio* collected after the winter 2002 food supplementation experiment in MZNP.

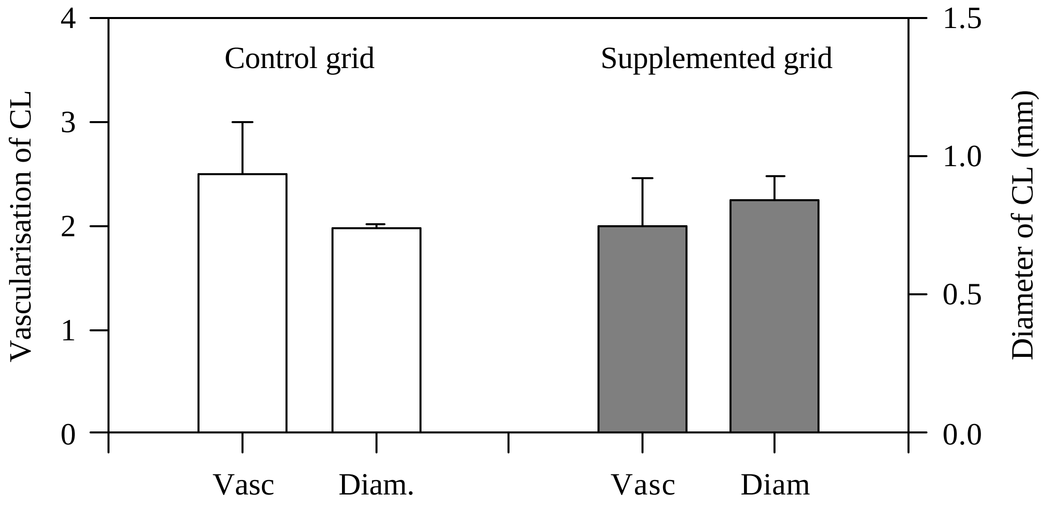

Figure 3.30: Mean (? 1SEM) vascularisation (Vasc) and diameter (Diam) of the largest corpus luteum per female *Rhabdomys pumilio* caught after the winter 2002 food supplementation experiment in MZNP.

The females from the supplemented grid had significantly thicker uterine walls ($P < 0.01$), endometria ($P = 0.02$) and myometria ($P < 0.01$), and significantly greater vascularisation of the uterine horns ($P = 0.02$) compared to the females from the control grid, however there was no significant difference in the number of glands present in the uterine walls of the females ($P = 0.74$; Figures 3.31, 3.32).

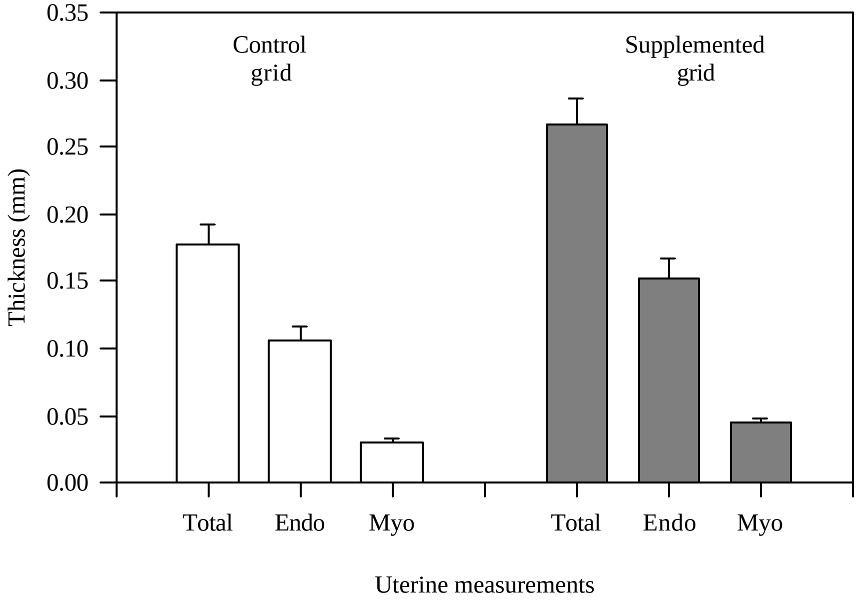

Figure 3.31: Mean measurements (? 1SEM) of the thicknesses of the uterine wall (Total), the endometrium (Endo) and myometrium (Myo) of female *Rhabdomys pumilio* caught after the winter 2002 food supplementation experiment in MZNP.

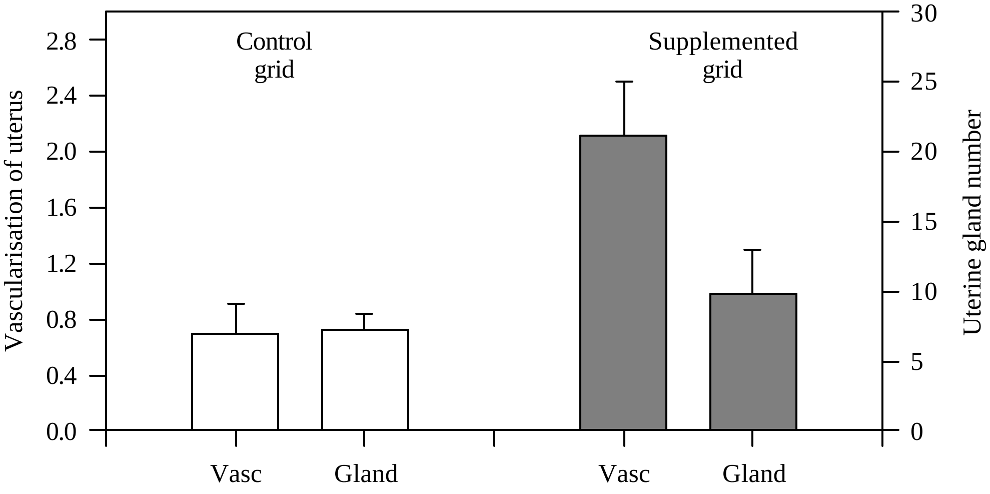

Figure 3.32: Mean (? 1SEM) vascularisation score (Vasc) and number of uterine glands (gland) for the uterine walls of female *Rhabdomys pumilio* caught after the winter 2002 food supplementation experiment in MZNP.

3.6.5) *Hormone assays:*

Plasma testosterone levels were not significantly different between the two grids (P = 0.472). Levels ranged between 11 and 480ng/dL, with the mean levels being 29.8 ? 5.38ng/dL for males from the control grid and 96.0 ? 50.45ng/dL for males from the supplemented grid. Levels of leptin-like immunoreactivity were not significantly different between the grids when the male and female values were combined (P = 0.815), however when the genders were compared within and across the grids it was evident that the females on the supplemented grid had significantly higher levels of leptin-like immunoreactivity than the males on the supplemented grid. (P = 0.032, Table 3.19).

Table 3.19: Mean levels of plasma leptin-like immunoreactivity for males and females from the control and supplemented grids in MZNP during the winter 2002 food supplementation experiment. Values are means ? 1 SEM.

	Supplemented grid	**Control grid**	**P value**
Females	12.08 ? 2.16^{a}	9.13 ? 2.63^{a,b}	0.032
Males	4.67 ? 0.71^{b}	8.22 ? 1.50^{a,b}	
Total	8.57 ? 1.45	8.70 ? 1.57	0.815

- within gender comparison (both within and between grids), the groups with different superscript letters (a or b) are significantly different ($P < 0.05$).

3.7) Discussion:

The three experiments were designed to test whether provision of supplementary food in winter would override the normal inhibition of reproduction that occurs at this time (Brooks, 1974; Perrin, 1980; David & Jarvis, 1985). The basic requirement for these experiments was therefore that reproduction would be inhibited in winter, however this did not occur in the first two experiments at Thomas Baines Nature Reserve. The most probable reason for this was that the winters were milder than is usual for the area and there was, therefore, not a sufficiently severe energetic challenge to inhibit reproduction. Although the two experiments at Thomas Baines Nature Reserve could be regarded as failures, they do further highlight the reproductive flexibility that characterises *Rhabdomys pumilio* and some aspects of these results will be discussed later. Two other fundamental requirements of the experimental design were that the mice ate the food and that there was no movement of mice between the grids. At Thomas Baines Nature Reserve there was indirect evidence that *Rhabdomys pumilio* consumed the bird seed. Although this might be seen as a weakness of the study, it is not important since reproduction was not inhibited on the control grid. By contrast, at MZNP there was direct evidence (observation of *Rhabdomys pumilio* feeding from the feeders and food dye in stomach contents) and indirect evidence (fragments of food along runways and at the opening of an occupied burrow) that *Rhabdomys pumilio* consumed some of the provided food. There was some movement of animals between the control and supplemented grids at Thomas Baines Nature Reserve in both experiments but this was minimal (less than 10% of the mice caught in any one trap session). At MZNP, there was no movement detected between the grids.

Although the experiments at Thomas Baines Nature Reserve did not generate the desired results, there were some effects of food supplementation that are worth discussing. In both 2000 and 2001, food supplementation resulted in a significant increase in the number of mice caught and estimated population size. Based on external assessment of reproductive condition, there were significantly more reproductively active mice on the supplemented grid in 2000 and 2001, and the mice on the supplemented grid were significantly heavier than those from the control grid for both years. Previous studies on a variety of species of mammals have produced similar results, showing that provision of supplementary food in an area results in an increase in the number of reproductively active animals (Cole & Batzli, 1978; Desy & Thompson, 1983), an increase in the number of pregnant females (Briggs, 1986; O'Donoghue & Krebs, 1992; Cittadino *et al.*, 1994; Galindo-Leal & Krebs, 1998), and an increase in body mass (e.g. Flowerdew, 1972; Havera & Nixon, 1980; Sullivan, 1990; Monadjem & Perrin, 1996). The increased number of mice caught on the supplemented grid could be due to increased immigration by animals attracted to the additional food, increased survival, an increase in litter size or the percentage of females pregnant, or a decrease in the inter-litter interval. The small number of mice caught in the middle line of traps suggests that immigration is an unlikely explanation and it is more likely that the additional food had a small positive effect on survival and litter size (Desy & Thompson, 1983; Briggs, 1986; Cittadino *et al.*, 1994).

In both experiments (2000 and 2001), the estimated populations of mice from both grids increased in size during the winter, rather than decreasing, as one would expect had reproduction been inhibited. This was probably due to the continuous production of litters, evidence for which was the continuous presence of juveniles in the population. In addition, females were significantly heavier than the males and were likely to be pregnant, as confirmed at dissection. Since the increase in population size occurred on both grids, it cannot be attributed to the supplemented food, and is more likely to be related to the climatic conditions. Interestingly, in 2000, juveniles were captured on the supplemented grid during each of the trapping sessions, while on the control grid in August no juveniles were caught. In 2001, 17 juveniles were trapped on the supplemented grid in June, which is more than all those caught on the control grid. This increased occurrence of juvenile *Rhabdomys pumilio* on the supplemented grid may be due the presence of supplementary food allowing for more litters to be produced and an increase in litter size and juvenile survival. Similar results have been reported by Cole and Batzli (1978), who found that by exposing *Microtus ochrogaster* to food supplementation, there was an increase in litter size and breeding intensity. In addition, this could also explain the increase in the number of mice caught on the supplemented grid.

In 2000, on both grids, there was a significant increase in the mean hind foot length of the mice over the experiment, suggesting that mice of all age groups were surviving and thus the populations were not only increasing in size but also included more old, larger mice. Similar results were achieved by Galindo-Leal and Krebs (1998) who noted that food supplementation had no effect on the survival rates of *Peromyscus difficilis*. In 2000, in spite of the increase in hind foot length, there was a significant decrease in body mass of adults on both grids. This probably indicates that although the winter was mild, it was a period of food shortage and that the supplementary food was not enough to counter this. By contrast, in 2001 the mean mass of all the mice on both grids increased significantly. This could be a result of the accumulative effect of two consecutive mild winters on natural food availability.

While the field data and external observations of reproductive condition suggest that provision of supplementary food had an effect, this was not the case with the data collected from the more detailed analyses of reproductive condition of the mice collected at the end of the experiment. In both 2000 and 2001 there was no indication of any inhibition of gametogenesis in mice from the control grid and no significant differences between any of the features measured across the grids. While these results could be a result of mice from the control grid moving across to the supplemented grid, so that in effect all mice were receiving additional food, this is unlikely since the mid-line of traps did not catch sufficient mice to support this. It is much more likely that the particularly mild winters in the two years allowed reproduction to continue on the control grid.

In the third experiment, in MZNP, the winter was typical for the region, with low maximum and minimum temperatures and frost, although it was wetter than usual towards the end of the experiment. Unlike the first two experiments, the estimated population sizes on both grids decreased through winter, as did the actual number of mice caught. Furthermore, provision of additional food had no effect on estimated population size. The reduction in estimated population size during the experiment is probably linked to the fact that juveniles were caught only in May. May is considered to be the end of the breeding season of *Rhabdomys pumilio* (Brooks, 1974; Perrin, 1980; Henschel *et al.*, 1982; David & Jarvis, 1985) and my results indicate a reduction in reproductive activity at the end of summer. Further support for the reduction in reproductive activity includes a significant decrease in the number of reproductively active mice on both grids over the experimental period and that males were significantly heavier than females indicating that the females were unlikely to be pregnant. The reduction in population size through the experiment could also be attributed to the loss of animals through death or emigration. The significant decrease in the size of the hind feet of sub-adults and adults through the experiment suggests that the larger animals of the population were either dying or emigrating. There was a significant reduction in the occurrence of reproductively active mice (assessed externally) through the experiment and there was a significant difference between the grids such that provision of additional food was associated with an increase in the occurrence of reproductively active mice. Although other researchers have reported that food supplementation has no effect on the survival of animals or on the decline of populations (Galindo-Leal and Krebs, 1998 - *Peromyscus difficilis*; Saitoh, 1989 - *Clethrionomys rufocanus*), this is in contrast to a number of other studies which have suggested that with an increase in food supply, populations experience improved survival (e.g. Desy & Thompson, 1983; Sullivan *et al.*, 1983; Briggs, 1986; McAdam & Millar, 1999), extended breeding periods (e.g. Hansen & Batzli, 1979; Vaughan & Keith, 1981; Saitoh, 1989; McAdam & Millar, 1999), an increase in immigration rates (e.g. Flowerdew, 1972; Havera & Nixon, 1980; Saitoh, 1989; Cittadino *et al.*, 1994) and a reduction in the typical population declines noted for the various species (Flowerdew, 1972; Cole & Batzli, 1978; Taitt & Krebs, 1983; Sullivan, 1990).

However, food did affect individual animals such that mice on the supplemented grid were significantly larger (larger hind feet) and significantly heavier than the mice on the control grid after supplementation occurred, and significantly more of them were reproductively active, based on external examination. The fact that mice were significantly larger (as compared to simply heavier) on the supplemented grid may be linked to an increase in the growth rate, since studies have indicated that, with an increase in food supplies, individuals will improve their growth rates (e.g. Vaughan & Keith, 1981; Sullivan *et al.*, 1983; Monadjem & Perrin, 1996; McAdam & Millar, 1999). Alternatively, the provision of supplemental food might increase survival of the older animals and thus result in a larger mean hind foot length on the supplemented grid. The change in body mass has also been demonstrated previously, whereby an increase in food availability resulted in animals increasing their body masses (e.g. Taitt & Krebs, 1983; O'Donoghue & Krebs, 1992; Neal, 1996; Nunes *et al.*, 2000) and similar results for the increase in the number of reproductively active animals were obtained when populations of *Microtus pennsylvanicus* (Desy & Tompson, 1983) and *Microtus ochrogaster* (Cole & Batzli, 1978) were exposed to food supplementation.

The histological results for the mice from the MZNP study yielded interesting results. Although mice from the supplemented grid had significantly larger reproductive organs and were therefore expected to be more reproductively active than the mice from the control grid, males from both grids were spermatogenically active. Ovarian activity was not affected by food supplementation but development of the uterus was, and the females from the supplemented grid had thicker and more vascularised uterine walls. A comparison of the size of the reproductive organs of females from the three field experiments (Table 3.20) shows that in the MZNP experiment, the uterine horns were substantially lighter and the walls less developed than those from the Thomas Baines Nature Reserve experiments. Based on this comparison, it is assumed that the females from MZNP were reproductively inactive.

Table 3.20: Comparison of the mean masses of the reproductive organs of male and female *Rhabdomys pumilio* collected during the three consecutive field experiments and the mean uterine measurements for the females. Values are means; standard errors are omitted from the table, but are included in the original graphs (Figures 3.7; 3.11; 3.17; 3.19; 3.26 & 3.31).

Measurements	**2000**		**2001**		**2002**	
	Control	**Supplement.**	**Control**	**Supplement**	**Control**	**Supplement**
?						
Testes (g)	0.65	0.62	0.59	0.76	0.62	0.90
Epid. (g)	0.19	0.17	0.16	0.22	0.15	0.23
Acc. gld. (g)	0.36	0.40	0.31	0.47	0.08	0.21
?						
Ovary (g)	0.04	0.03	0.04	-	0.02	0.03
Uterus (g)	0.13	0.11	0.54	-	0.04	0.08
Uterus:						
Total (mm)	0.67	0.55	0.36	-	0.18	0.27
Endometrium (mm)	0.28	0.28	0.19	-	0.11	0.15
Myometrium (mm)	0.12	0.07	0.07	-	0.03	0.05

By contrast, the reproductive organs of the males from the three experiments were of a similar size. These results indicate that despite energetic challenges, male *Rhabdomys pumilio* will remain spermatogenically active, while females will slow or cease their reproductive activity in response to reduced ambient temperatures regardless of the food availability. This observation suggests that it is the females that control the breeding season, while males remain reproductively ready despite energetic challenges (Swanepoel, 1980; Clarke, 1985; Hamilton & Bronson, 1985; Bronson & Perrigo, 1987; Bronner *et al*., 1988; Bronson & Heideman, 1994).

Overall, the results from all the field experiments indicate that food supplementation had little effect on the reproduction of *Rhabdomys pumilio*. In Thomas Baines Nature Reserve, it appears that the climate was mild enough to allow the mice to continue reproduction either in response to a natural increase in food availability, or due to the temperatures being mild or both. In MZNP, the climate also appears to have affected reproduction, however in this area a decline in reproductive activity occurred despite the presence of supplementary food. Since the provision of extra food did not override the energetic challenge of winter, it is possible that *Rhabdomys pumilio* in the Cradock region are in fact responding to photoperiodic cues. Although mice from Thomas Baines Nature Reserve do not respond to photoperiodic cues in the laboratory (Jackson & Bernard, 1999), the potential use of photoperiod in Cradock is not an unreasonable suggestion, since the climate in this area is more predictable and is harsher than the climate in the Grahamstown region. Bronson and Perrigo (1987), Bronson and Heideman (1994), and Demas and Nelson (1998) have indicated that populations of the same species, in various areas can respond differently to the same environmental cues.

Although food supplementation did not affect the reproductive activity of *Rhabdomys pumilio*, the addition of food to the environment did improve the condition of these mice by resulting in an increase in body mass and rate of growth. It is therefore evident that the reproductive activity of *Rhabdomys pumilio* is not controlled by food alone and that other environmental factors influence reproduction in these mice. It has previously been reported that male and female *Rhabdomys pumilio* respond differently to energetic challenges under laboratory conditions (Jackson & Bernard, 2001) and the results from the field experiments confirm this, where the females contribute to the control of the breeding season while the males remain reproductively ready.

CHAPTER FOUR: EFFECTS OF EXOGENOUS GNRH ON THE REPRODUCTIVE ACTIVITY OF MALE *RHABDOMYS PUMILIO* EXPOSED TO LOW AMBIENT TEMPERATURES AND LOW FOOD AVAILABILITY.

The reproductive axis of mammals is controlled by a variety of hormones, the most important of which is the decapeptide, gonadotropin-releasing hormone (GnRH). GnRH is produced by neurosecretory cells in the hypothalamus (Raga *et al*., 1999; Vizcarra *et al.*, 1999; Marshall *et al.*, 2001) and plays a crucial role in regulating reproductive function and the development of the gonads (Raga *et al.*, 1999; Parvizi, 2000). GnRH, which is released in a pulsatile manner (Rhim *et al.*, 1993; Hammilton *et al.*, 1999; Vizcarra *et al.*, 1999), is responsible for stimulating production of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Garrel *et al.*, 1998; Raga *et al*., 1999; Marshall *et al*. 2001) and, in turn, these two hormones support the development of the gonads, gametogenesis and the production and release of the gonadal steroids (Garrel *et al.*, 1998; Behr & Weinbauer, 1999). The release of GnRH and thus of the gonadotropins is controlled in part by feedback mechanisms, whereby the gonadal steroids, such as testosterone and progesterone, reduce the pulsatile release of GnRH and thus LH and FSH (Perheentupa *et al.*, 1995), and reduce the responsiveness of the pituitary gland to GnRH (see Bronson, 1986; Rhim *et al.*, 1993).

In addition, a number of physiological states and hormones other than the gonadal steroids affect GnRH production and therefore reproductive activity. Cortisol, which is produced in response to a number of different stressors, reduces plasma LH and FSH levels (see Dubey & Plant, 1985) and suppresses oestradiol secretion (Daley *et al.*, 1999). It has been suggested that cortisol acts at hypothalamic and, or hypophyseal sites to reduce GnRH secretion (Dubey & Plant, 1985; Daley *et al.*, 1999). Studies using sheep and monkeys have indicated that the negative effects of cortisol can be overridden by episodic or pulsatile administration of GnRH, causing an increase in the release of the gonadotropins and a reactivation of follicular development and ovulation (Dubey & Plant, 1985; Daley *et al*., 1999). Prolonged exercise has also been shown to reduce reproductive activity in a range of mammals from mice to humans (Frisch, 1984; Manning & Bronson, 1991) and infusion of GnRH induces normal oestrous cyclicity and ovulation in exercised rats (Manning & Bronson, 1991). Many studies of the control of reproduction have examined the effects of food restriction on reproductive activity. In these studies it has been found that with a reduction in food availability there is a decrease in GnRH production and a reduction in LH and FSH release (Bronson, 1988; Bergendahl *et al.*, 1991; Perheentupa *et al.*, 1995; Mounzih *et al.*, 1997), and in females, a reduction in food availability results in anoestrus (e.g. in cows - Hamilton *et al.*, 1999). In all cases, pulsatile administration of GnRH reverses the adverse effects of food restriction, and LH secretion increases with a resumption of ovulation in females (Bronson, 1986; Gill & Rissman, 1997; Vizcarra *et al.*, 1997; Hamilton *et al.*, 1999) and testicular androgen production in males (Bergendahl *et al.*, 1991).

Recently it has been suggested that nitric oxide (NO) can stimulate the secretion of GnRH (Bhat *et al.* 1995 cited by Dixit & Parvizi, 2001). Similarly, there are other compounds which can affect GnRH production, including leptin (produced by adipocytes), which can directly influence GnRH, LH and FSH release (Yu *et al.*, 1997a; Lebrethon *et al.*, 2000; Dixit & Parvizi, 2001) or indirectly increase GnRH production by activating NO production (Yu *et al.*, 1997b), carbon monoxide (CO), which has been implicated in stimulating GnRH release (Lamar *et al.*, 1996), and GnRH analogs which can stimulate the recovery of gametogenesis (Shuttlesworth *et al.*, 2000).

The results of these studies indicate that the responses of the reproductive axis to stressful situations are mediated by the hypothalamus, resulting in a suppression of GnRH release, either through a reduction in pulse frequency or pulse amplitude. It has been suggested that the pituitary gland may also be affected, where there is an impairment of the pituitary function, resulting in a reduction of gonadotropin release (Bennett *et al.*, 1993; Bennett *et al.*, 1997). However, I'Anson *et al.* (2000) suggest that the sensitivity of the pituitary gland to stressful situations could be altered in some species of animal, while in other species it may not be affected.

It is likely that the winter inhibition of reproduction in *Rhabdomys pumilio* is controlled at the level of the hypothalamus by a change in the pattern of release of GnRH, possibly brought about by an energetic challenge working through cortisol and, or leptin. Thus, the aim of the following experiment was to determine whether exogenous GnRH would override the inhibitory effects of an energetic challenge on the reproductive activity of male *Rhabdomys pumilio*.

4.1) Materials and methods:

Eighteen male, adult, *Rhabdomys pumilio* were trapped in Thomas Baines Nature Reserve during October (early summer) 2000, when the mice are typically reproductively active (pers. obs.), brought into the laboratory and placed into a controlled environment (CE) room. The mice were housed individually in cages of similar size (41x26x15 cm) and were each provided with shredded paper and a square of blanket for nesting material and a toilet roll for cover. Food (rabbit pellets, Epol, Johannesburg (JHB), RSA.) and water were provided *ad libitum.* Cages were cleaned at the end of each week.

4.1.1) *Acclimation:*

Mice were acclimated for two weeks at 15°C (typical winter temperature) with a photoperiod of 12L:12D and a humidity of around 40%. During this time the mice were maintained on an *ad libitum* diet of rabbit pellets. Food was weighed before being given to the mice and during the second week of the acclimation period all the remaining uneaten food for that week was collected and used to calculate the food consumed during that week, and from this, the daily food consumption for each mouse. Mice were weighed every Monday and Thursday and the position of the testes (scrotal, moving-scrotal or non-scrotal) was noted. At the end of the acclimation period, the mean mass of each mouse was calculated using the weights recorded during the last week of acclimation.

4.1.2) *Food restriction:*

After the two-week acclimation period, all the mice were exposed to a food-restricted diet where mice were subjected to a 10% reduction in their daily food intake. This reduction in food intake, combined with a low ambient temperature (15°C), will inhibit spermatogenesis (Jackson & Bernard, 2001). The period of food restriction continued for four weeks, at the end of which the mice were either non-scrotal and assumed to be reproductively inactive (Jackson & Bernard, 2001), or showing signs of becoming non-scrotal (testes either partly-scrotal or small while still in the scrotal sacs). Throughout the period of food restriction, mice were weighed and the position of the testes assessed every second day. If at any stage during food restriction the body mass of a mouse dropped below 70% of the mean mass calculated during the last week of acclimation, the daily food provided was increased so that it received the daily food intake less 1 to 5%. This continued until the body mass was above the 30% reduction mark.

4.1.3) *Hormonal manipulation:*

After the four-week period of food restriction, the mice were divided into two groups (one of ten animals and the other of eight) such that the mean body mass of each group and the reproductive status of the mice in each group were similar. The group of ten mice were administered with 1?g GnRH (Kindly donated by the MRC Regulatory Peptides Research Unit; Department of Chemical Pathology, University of Cape Town Medical School, R.S.A) in 200?l of saline twice a day (7h00 and 19h00) for 21 days, while the group of eight mice received 200?l of saline at the same times. Injections were administered subcutaneously. All other conditions remained as they had been during the period of food restriction, and the mice were weighed and the position of the testes assessed every second day. Twenty-one days of exogenous GnRH was felt to be sufficiently long to stimulate spermatogenesis and thus bring about a visible change in the histology of the testes (Setchell, 1982). The dosage of GnRH used in the current experiment was based on work by N. Bennett (Department of Zoology and Entomology, University of Pretoria, R.S.A, pers. comm.; Bennett *et al.*, 1993).

4.1.4) *Sacrifice, tissue collection and blood collection:*

At the end of the period of hormone manipulation, the mice were sacrificed, weighed, dissected and given a subjective fat index as previously described (Chapter 3). Blood was collected and processed as previously described.

4.1.5) *Histological analysis:*

Reproductive organs were processed and spermatogenic activity and the activity of the accessory glands assessed as previously described (Chapter 3).

4.1.6) *Hormone assays:*

Plasma testosterone levels were determined as previously described (Chapter 3). Plasma LH was measured using a rat luteinizing hormone EIA (Amersham Biotrak, UK). The kit uses plate strips coated with rabbit anti-rat LH, standards of rat luteinizing hormone and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution as the stain. Sensitivity is 0.1ng/ml and inter and intra-assay variances were less than 26%. It was not possible to validate the LH assay for *Rhabdomys pumilio* because of a shortage of plasma and in the results and discussion levels of LH-like immunoreactivity are reported.

4.1.7) *Statistical analysis:*

Statistical analyses were conducted using both SigmaStat and Statgraphics. Changes in the body weight of individual mice were compared with a repeated measures t-test, while comparisons between the experimental groups were conducted using Student's t-tests and non-parametric equivalents where applicable. Percentages were arcsine transformed for statistical analysis, however these data are presented as percentages within tables. Differences were considered significant at a < 0.05 and values are expressed as means ± 1 SEM, unless otherwise stated.

4.2) Results:

Before dissection, one mouse from each group died and only mice that survived to dissection were included in the analyses. At the start of the experiment, prior to food restriction and hormone manipulation, the eighteen mice were of similar body mass. During food restriction and the hormone manipulation period, there was a statistically significant reduction in body mass of all the mice (P < 0.05). However, there was no significant difference in the mean body masses of the two groups at the start or at the end of the hormone manipulation period (P > 0.05; Table 4.1).

Table 4.1: The effect of exogenous GnRH on body mass at dissection and mass of the reproductive organs of *Rhabdomys pumilio* subjected to food restriction and a low ambient temperature. Body mass data at the start of the hormone manipulation period have been included. Values are means ? 1SEM.

	Mean masses (g)		**P value**
	GnRH treated mice	**Saline treated mice**	**(t-tests)**
Body: start	42.50 ? 2.54	43.90 ? 3.45	0.731
end	34.65 ± 1.29	35.31 ± 1.98	0.775
Testes	0.70 ± 0.04	0.40 ± 0.08	0.006
Epididymides	0.21 ± 0.02	0.14 ± 0.02	0.026
Accessory gland	0.24 ± 0.03	0.17 ± 0.03	0.083

The mice were divided into the two experimental groups so that there was a similar distribution of scrotal, partly-scrotal and non-scrotal mice (Table 4.2). At dissection, four of the control (saline-treated) mice were non-scrotal and the remaining mice were partly-scrotal, while in the GnRH treated group, four of the mice were non-scrotal, three were partly-scrotal and two were scrotal.

Table 4.2: The reproductive status of mice in the two experimental groups at the start and at the end of the hormone manipulation period of the GnRH experiment. (NS = non-scrotal; PS = partly scrotal; S = scrotal).

	Number of mice					
	Start			**End**		
	NS	**PS**	**S**	**NS**	**PS**	**S**
Control	4	3	0	4	3	0
GnRH	4	4	1	4	3	2

4.2.1) *Dissection data:*

At dissection the mean masses of the testes and epididymides of the mice treated with GnRH were significantly greater than those of the control mice ($P = 0.006$ and $P = 0.026$ respectively; Table 4.1), while there was no significant difference in the mean mass of the accessory glands ($P = 0.083$). Examination of the bodies of the mice at dissection revealed that only one individual from the GnRH treated group had some body fat (fat index = 1) while the others were lean (fat index = 0).

4.2.2 *Histology:*

Histological analysis of the reproductive organs indicated that all of the GnRH-treated mice were spermatogenically active, while four of the seven saline-treated mice were spermatogenically active, with above 80% of the seminiferous tubules producing spermatozoa. The mice treated with GnRH were more spermatogenically active with higher epididymal sperm storage and storage of secretory material in the accessory glands compared to the saline-treated mice (Table 4.3). However, statistical analysis indicated that there were no significant differences in the activities of the organs of the two groups ($P > 0.05$ in all cases; Table 4.3).

Table 4.3: The effect of exogenous GnRH on the activity of the reproductive organs of *Rhabdomys pumilio*. Values are means ? 1 SEM.

	Mean values for the groups (%)		**P value**
	GnRH treated mice	**Saline treated mice**	**(t-tests)**
Spermatogenic activity (testes)	89.3 ± 1.70	53.3 ± 17.7	0.266
Sperm storage (epididymides)	85.3 ± 4.81	47.3 ± 16.1	0.050
Activity of the Accessory glands	96.3 ± 3.67	73.9 ± 9.52	0.098

4.2.3 *Hormone assays:*

There were no significant differences in levels of plasma LH-like immunoreactivity or plasma testosterone levels between the two experimental groups ($P > 0.05$; Table 4.4). Within the saline treated group, there was no statistically significant difference between the mean plasma testosterone levels of the four spermatogenically active mice (? = 26.4 ± 10.15) and the three inactive mice (? = 11.2 ± 1.17).

Table 4.4: The effect of exogenous GnRH on plasma testosterone and LH-like immunoreactivity levels of *Rhabdomys pumilio*. Values are means ± 1SEM.

Hormone	**Treatment**		**P value**
	GnRH	**Saline (control)**	**(t-test)**
Testosterone (ng/dL)	20.44 ± 3.94	19.86 ± 6.25	0.56
LH (ng/ml)	0.58 ± 0.13	0.51 ± 0.09	0.63

4.3) Discussion:

It has been shown that both a reduction in food availability and ambient temperature inhibit spermatogenesis in *Rhabdomys pumilio* (Jackson & Bernard, 2001). Such environmental factors are thought to act primarily at the hypothalamic level, altering the secretion of GnRH and thus affecting activity of the gonads (Miller *et al*., 1998). An acute reduction in food in rats, for example, causes a reduction in the synthesis and secretion of GnRH and therefore the gonadotropins LH and FSH (see Perheentupa *et al.,* 1995; Bronson, 1988; Bergendahl *et al.*, 1991). Since ambient temperature will affect the metabolism of an endothermic and homeothermic animal (Bronson & Perrigo, 1987; Bronner *et al.*, 1998; Bronson & Heideman, 1994), it is likely that a reduction in temperature will also affect the hypothalamus and disrupt the secretion of GnRH. Furthermore, it is probable that the two environmental cues will be synergistic in creating an energetic challenge.

The results from the current experiment have shown that mice that received exogenous GnRH for 21 days had significantly larger testes and epididymides and, although the differences were not statistically significant, reproductive organs that were more active. While the results are equivocal, there is a clear indication that treatment with exogenous GnRH did override the inhibitory effects of the experimental reduction in food availability and ambient temperature. Similar results have been reported in various studies on other animals, including rats (Bergendahl *et al.* 1991; Manning and Bronson, 1991; Perheentupa *et al.* 1995), gilts (Miller *et al.* 1999) and cattle (Hamilton *et al.* 1999). However, most of these studies have reported that infusion or injection with GnRH results in an increase in the levels of LH, testosterone, and to a lesser extent, FSH (Vizcarra *et al.* 1999; Perheentupa *et al.* 1995). The results of the present study suggest that exogenous GnRH did not affect levels of LH-like immunoreactivity or testosterone but at the same time did affect the size of the testes and accessory glands.

A fundamental assumption in this experimental design was that after four weeks of energetic challenge, the mice would be spermatogenically inactive, with low levels of GnRH, and that provision of exogenous GnRH would initiate a new cycle of spermatogenesis. However the energetic challenge did not completely inhibit spermatogenesis and four of the mice from the control group that had been exposed to an energetic challenge for a total of seven weeks, were spermatogenically active. It is thus perhaps not surprising that exogenous GnRH failed to have the expected results.

Non-breeding adult female naked mole rats (*Heterocephalus glaber*) are insensitive to doses of exogenous GnRH of less than 1.0?g per animal (Faulkes *et al*., 1990) and it is possible that a similar lack of sensitivity explains the results from the present study. If the anterior pituitary of *Rhabdomys pumilio* had been insensitive to GnRH, this would explain why GnRH had no effect on plasma LH or testosterone levels or spermatogenesis. It is also possible that by not administering GnRH in a pulsatile manner, as is the case with endogenous GnRH, the hormone pathways were not fully stimulated. However, neither of these arguments explains why the GnRH-treated animals had significantly heavier reproductive organs.

In conclusion there is evidence that exogenous GnRH will partially counter the inhibitory effects of an energetic challenge on spermatogenesis. This suggests, as has been shown in other mammals, that in *Rhabdomys pumilio* the energetic challenge inhibits spermatogenesis by acting at the hypothalamic level and affecting GnRH secretion.

CHAPTER FIVE: THE EFFECTS OF EXOGENOUS LEPTIN ON THE REPRODUCTIVE ACTIVITY OF MALE *RHABDOMYS PUMILIO* EXPOSED TO LOW AMBIENT TEMPERATURES AND LOW FOOD AVAILABILITY.

In the 1950s it was believed that food intake was controlled by either a thermo-sensitive mechanism or a chemo-sensitive mechanism, however even at this early stage the latter concept was more accepted (Kennedy, 1953). More recently, work on the genetically obese (*ob/ob*) mouse has resulted in the identification of the *ob* gene and the product of this gene (Zhang *et al.*, 1994). This product has become known as leptin (from the Greek root *leptos* meaning thin; Caro *et al.*, 1996) and is thought to act as an "adipostat," carrying information regarding the size of energy reserves in the body in mammals (Zhang *et al.*, 1994; Houseknecht & Portocarrero, 1998; Cunningham *et al.*, 1999) and birds (Denbow *et al.*, 2000).

Leptin is a 16kDa (Chehab, 1997; Tartaglia, 1997; Barb *et al.*, 2001), 167-amino acid protein (Zhang *et al.*, 1994; Auwerx & Staels, 1998; Cunningham *et al.*, 1999) produced by the obese (*ob*) gene (Barash *et al.*, 1996; Mounzih *et al.*, 1997; Yu *et al.*, 1997a) and is mainly produced by white adipose tissue (Flier, 1997; McCann *et al.*, 1998; Gonzalez *et al.*, 2000). More recently it has been discovered that leptin is produced by other tissue types including brown adipose tissue (Auwerx & Staels, 1998; Atcha *et al.*, 2000) and placental tissue (Chehab, 1997; Ashworth *et al.*, 2000; Henson & Castracane, 2000). There are five or more isoforms of leptin receptors that are generally referred to as either long-form (OB-Rb) or short-form (OB-Ra) receptors (Flier, 1997; Barkan *et al.*, 1999; Cunningham *et al.*, 1999), both of which belong to the class I cytokine receptor superfamily (Barkan *et al.*, 1999). The long-form receptors, the likely signalling forms (Tartaglia, 1997), are predominantly expressed in the hypothalamus (Barkan *et al.*, 1999), which is regarded as the main target centre of leptin (Howlett, 1996; Nagatani *et al.*, 1998; Atcha *et al.*, 2000; Sansinanea *et al.*, 2001). The short-form receptor is expressed in various tissues (Barkan *et al.*, 1999) including the lung, kidney and choroid plexus in the brain (Flier, 1997; Tartaglia, 1997). Other regions in which leptin and its receptors are expressed include the pituitary gland (McCann *et al.*, 1998; Clarke & Henry, 1999; Tena-Sempere *et al.*, 1999), where leptin receptors are expressed predominantly by the TSH cells (Jin *et al.*, 2000), the gonads (Clarke & Henry, 1999; Tena-Sempere *et al.*, 1999; Henson & Castracane, 2000), uterus (Henson & Castracane, 2000), fundic gastric epithelium (Bado *et al.*, 1998), skeletal muscle (Wang *et al.*, 1998), and the liver (Chen *et al.*, 1996).

The production of leptin appears to be mediated by a variety of hormones and other chemicals. These include glucose (Foster & Nagatani, 1999) and insulin (Houseknecht & Portocarrero, 1998; McManus & Fitzgerald, 2000; Barb *et al.*, 2001), glucocorticoids (Masuzaki *et al.*, 1997; Bai & Castonguay, 2001; Moschos *et al.*, 2002), cocaine- and amphetamine-regulating transcript (CART; Lebrethon *et al.*, 2000), steroid hormones (Henson & Castracane, 2000), MSH and its receptor (Fan *et al.*, 1996 and Huszar *et al.*, 1997 – cited by Auxerx & Staels, 1998), nitric oxide (NO; Rettori *et al.*, 1993), glucosamines (Wang *et al.*, 1998) and neuropeptide-Y (NPY; Yu *et al.*, 1997a; Aubert *et al.*, 1998; Vuagnat *et al.*, 1998).

Initially, leptin was considered to control only food intake and to limit obesity (Ahima *et al.*, 1996; Flier, 1997; Houseknecht & Portocarrero, 1998), whereby an increase in circulating leptin would decrease food intake and body mass (Ahima *et al.*, 1996; Muzzin *et al.*, 1996; Buison *et al.*, 2001). Evidence for this regulation was obtained when genetically obese (*ob/ob*) mice, which had a mutation on the *ob* gene which resulted in a lack of leptin production, were administered with recombinant *ob* product (leptin) and responded by reducing their food intake and body mass (Campfield *et al.*, 1995; Barash *et al.*, 1996). However, it was observed that these *ob/ob* mice were also sterile and on receiving recombinant leptin they became reproductively active (Barash *et al.*, 1996; Chehab *et al.*, 1996; Cunningham *et al.*, 1999). Since this finding, leptin has been regarded as also playing a role in the regulation of reproductive activity (Yu *et al.*, 1997a; Aubert *et al.*, 1998; McCann *et al.*, 1998). Treatment with exogenous leptin will result in an increase in LH levels in *ob/ob* mice (Barash *et al.*, 1996), normal mice (Henson & Castracane, 2000), rats (Yu *et al.*, 1997a & b; Nagatani *et al.*, 1998) and monkeys (Cunningham *et al.*, 1999), which may be due to leptin acting on the hypothalamus stimulating the release of GnRH (Clarke & Henry, 1999; Foster & Nagatani, 1999; Moschos *et al.*, 2002) and therefore an increase in LH production, or acting directly on the pituitary (Nagatani *et al.*, 1998; Clarke & Henry, 1999; Cunningham *et al.*, 1999; Tena-Sempere *et al.*, 1999).

Leptin not only acts as a signal between the nutritional status of an animal and its reproductive axis (Mounzih *et al.*, 1997; Barkan *et al.*, 1999; Cunningham *et al*, 1999) but also regulates the onset of puberty (Chehab *et al.*, 1997; Atcha *et al.*, 2000; Almog *et al.*, 2001). Leptin has also been implicated in playing a role during pregnancy (Holness *et al.*, 1999; Ashworth *et al.*, 2000; Moschos *et al.*, 2002) and placental leptin is regarded as being important for foetal growth and development (Holness *et al.*, 1999; Ashworth *et al.*, 2000). In addition to these factors, leptin also accelerates behavioural oestrus and mating (Chehab *et al.*, 1997), affects bone metabolism

(Moschos *et al.*, 2002), increases neural activity (Shiraishi *et al.*, 2000), stimulates GH secretion (Vuagnat *et al.*, 1998), controls thyroid and adrenal steroid production (Ahima *et al*., 1996), triggers gonadotropin secretion (Barkan *et al.*, 1999; Moschos *et al.*, 2002, Yu *et al.*, 1997b), affects NO release (Yu *et al.*, 1997a) and affects the ovulation process (Clarke & Henry, 1999; Duggal *et al*., 2000).

Despite all the evidence indicating that leptin has an effect on the reproductive activity of mammals, most of the studies conducted on this subject have focussed on genetically obese mammals and little has been done on the effects of leptin on non-obese mice from natural populations rather than laboratory-bred animals. In a previous experiment, where spermatogenically active *Rhabdomys pumilio* were exposed to low ambient temperatures and low food availability, it was found that mice with larger stores of body fat had remained spermatogenically active despite the unfavourable conditions (Jackson & Bernard, 2001). What caused this persistence of reproductive activity despite the energetic challenge is not fully understood, but it was felt that there could be a link between the body fat reserves and the reproductive status of an individual. Leptin is the obvious link between the body fat reserves and reproduction in *Rhabdomys pumilio* and hence it was hypothesised that supplying mice that were exposed to an energetic challenge with exogenous leptin would override the inhibitory effect of the energetic challenge and the mice would remain spermatogenically active.

Thus, the aim of this study was to further examine the link between body fat stores and reproductive activity in male *Rhabdomys pumilio*.

EFFECTS OF SHORT-TERM TREATMENT WITH LEPTIN ON THE REPRODUCTIVE ACTIVITY OF MALE *RHABDOMYS PUMILIO*.

Because leptin is very expensive, a short-term experiment was designed to test if treatment of spermatogenically inactive *Rhabdomys pumilio* with exogenous leptin would result in an increase in plasma LH levels. Although it was unlikely that such a short experiment would affect the mass and histology of the reproductive organs, a full set of analyses (details below) were included.

5.1) Materials and methods:

Ten adult, male *Rhabdomys pumilio* were trapped in Thomas Baines Nature Reserve during October (early summer) 2000. The mice were brought into the laboratory and were placed into a CE room that was set at 15^{o}C, with a photoperiod of 12L:12D. The mice were housed individually in similar sized cages (41x26x15 cm) and were each provided with shredded paper and a square of blanket for bedding, and a toilet roll for cover. Food (Rabbit pellets, Epol, JHB, RSA) and water were provided *ad libitum*.

5.1.1) *Acclimation:*

Once all ten mice were caught, they were allowed to acclimate for two weeks in a CE room at 15^{o}C with a photoperiod of 12L:12D and a humidity of about 40%. The mice were maintained on an *ad libitum* diet of rabbit pellets and water, however, the food was weighed before being given to each mouse. The cages were cleaned once a week, and during the second week of acclimation, all remaining uneaten food for that week was collected and used to calculate the mean daily food intake for each individual mouse. The mice were weighed every Monday and Thursday and their reproductive status was noted, based on the position of the testes (scrotal, partly- or moving-scrotal, or non-scrotal). At the end of the acclimation period, the mean body mass, using the last three masses recorded for the mice, was calculated for each mouse.

5.1.2) *Food restriction:*

After the two-week acclimation period all of the mice were exposed to a food-restricted diet where they were subjected to a 10% reduction in their daily food intake. This reduction in food intake at 15^{o}C has been shown to inhibit spermatogenesis in *Rhabdomys pumilio* (Jackson & Bernard, 2001). Food restriction continued for four weeks during which the mice were weighed and their reproductive status was assessed every second day, and cages were cleaned once a week.

5.1.3) *Hormonal manipulation:*

After the four-week period of food restriction, all mice were non-scrotal and assumed to be spermatogenically inactive. At this stage mice were divided into two groups of five mice each, such that the mean body mass of the two groups was similar. All the animals continued to receive the food-restricted diet and were exposed to an ambient temperature of 15°C and 12L:12D photoperiod. One group of five each received injections of leptin (40?g; Sigma, St. Louis, U.S.A; Mouse recombinant expressed in *E. coli*) in 200?l of saline and the other five mice were injected with 200?l of saline. The dosage of leptin used was based on dosages utilized in other studies, which yielded positive interactions between exogenous leptin and reproductive function (Ahima *et al.*, 1996; Barash *et al.*, 1996). The injections were administered subcutaneously twice daily at 07h00 and 19h00, and the period of treatment lasted four days. The mice were weighed and their reproductive status was assessed every second day.

5.1.4) *Sacrifice, tissue collection and blood collection:*

At the end of the four-day period of treatment, the mice were sacrificed by subcutaneous injection of 0.2ml of Euthanaze. They were then weighed and the testes, epididymides and accessory were removed, weighed to the nearest 0.001g and placed into Bouin's fixative. Blood was collected from the heart by ventricular puncture and was processed and stored as previously described (Chapter 3, 3.1.2). Mice were given a subjective fat index from 0-3 (Jackson & Bernard, 2001).

5.1.5) *Histological analysis:*

Reproductive organs were sectioned and assessed as previously described (Chapter 3, 3.13; 3.1.3.1).

5.1.6) *Hormone assays:*

Plasma LH levels were assessed using an rLH EIA system as described in Chapter 4 (4.1.6) Plasma leptin levels were assessed using a Competitive Human Leptin EIA (Neogen Corporation, USA). The EIA uses goat anti-rabbit antibodies to capture specific leptin complexes. The sensitivity of the kit is 0.488ng/ml, the range detection is from 0.488ng/ml to

500.0ng/ml and the inter- and intra-assay variation is less than 11%. Since it was not possible to validate either assay kit for *Rhabdomys pumilio*, hormone levels will be referred to as levels of hormone-like immunoreactivity.

5.1.7) *Statistical analysis:*

Statistical analyses were conducted using both SigmaStat and Statgraphics. Individuals were compared with a repeated measures t-test, while comparisons between the experimental groups were conducted using Student's t-tests or the non-parametric equivalents where applicable. Percentages were arcsine transformed for statistical analysis however these data are presented as percentages within tables. Differences were considered significant at a < 0.05 and values are expressed as means ± 1 SEM, unless otherwise stated.

5.2) Results:

During the experiment, one of the saline-treated mice died and consequently all data for the control group for the start of the experiment were from five mice, while data from the end of the experiment were for four mice.

At the start of the period of hormone manipulation the mean body masses of the two groups were not significantly different ($P = 0.984$; Table 5.1) and all the mice were non-scrotal. During the period of hormonal manipulation there was no significant change in the body mass of the mice ($P > 0.05$) and at the end of the experiment the mean masses of the two groups were still similar ($P = 0.728$; Table 5.1).

5.2.1) *Dissection data:*

At dissection, two of the saline-treated (control) mice were non-scrotal and the other two were partly-scrotal, while four of the leptin-treated mice were non-scrotal and one was scrotal. In both control and experimental groups, all but one mouse per group had no body fat, while the two with fat had a score of 1. The mean mass of the testes, epididymides and accessory glands were all higher in the saline group (Table 5.1), however these differences were not statistically significant ($P > 0.05$).

Table 5.1: The effect of short-term treatment with leptin on body mass and on the masses of the reproductive organs of *Rhabdomys pumilio*. Body masses are for the start and end of hormone treatment; masses of the reproductive organs were recorded at sacrifice. All values are means ? 1 SEM.

		Mean masses (g)		**P-value (t-tests)**
		Leptin treated mice	**Saline treated mice**	
Body mass	Start	40.20 ? 4.00	40.30 ? 4.03	0.984
	End	39.84 ? 3.88	42.05 ± 4.82	0.728
Testes mass		0.65 ± 0.09	0.73 ± 0.14	0.640
Epididymides mass		0.21 ± 0.05	0.26 ± 0.07	0.603
Accessory gland mass		0.28 ± 0.08	0.31 ± 0.08	0.824

5.2.2) *Histology:*

Histological analysis of the reproductive organs indicated that all of the mice were reproductively active, with above 80% spermatogenic activity, 50% sperm storage in the epididymides and 60% activity of the accessory glands. Statistical analysis indicated that there were no statistically significant differences in the levels of organ activity (P > 0.05 in all cases; Table 5.2).

Table 5.2: The effect of short-term treatment with leptin on the activity of the reproductive organs of male *Rhabdomys pumilio*. All values are means ? 1SEM.

	Mean values for the groups (%)		P-value (t-tests)
	Leptin treated mice	**Saline treated mice**	
Spermatogenic activity (testes)	90.0 ± 1.38	86.5 ± 1.19	0.105
Sperm storage (epididymides)	80.2 ± 7.43	84.5 ± 11.2	0.749
Accessory gland activity	93.4 ± 6.60	100.0 ± 0.00	0.730

5.2.3) *Hormone assays:*

Short-term treatment with leptin did not have a significant effect on levels of plasma LH-like or leptin-like immunoreactivity (P > 0.05; Table 5.3). Leptin-treated mice had higher LH levels than the control mice, while the control mice had the higher leptin levels (Table 5.3).

Table 5.3: Effects of short-term treatment with leptin on levels of plasma LH-like and leptin-like immunoreactivity. Values are means ? 1SEM.

Hormone	Treatment		P-value (t-tests)
	Leptin	**Saline (control)**	
Leptin (ng/ml)	64.48 ± 34.46	180.24 ± 112.95	0.408
LH (mIU/ml)	0.66 ± 0.15	0.56 ± 0.14	0.628

EFFECTS OF LONG-TERM TREATMENT WITH LEPTIN ON THE REPRODUCTIVE ACTIVITY OF MALE *RHABDOMYS PUMILIO*.

Having secured a donation of leptin (Amgen, California, U.S.A.), a long-term experiment was planned. Since it is the inhibition of spermatogenesis, rather than the initiation of a new cycle of spermatogenesis, that is of interest, the experiment was designed to establish if exogenous leptin, presented concurrently with an energetic challenge, would override the inhibitory effect of an energetic challenge on spermatogenesis.

5.3) Materials and methods:

Rhabdomys pumilio were collected from Thomas Baines Nature Reserve during February 2002 (late summer), during which time mice in the wild are typically reproductively active (Brooks, 1974; pers. obs.). Twenty-three male *Rhabdomys pumilio* were trapped and were brought into the laboratory where they were placed into a CE room set at 26°C, with a photoperiod of 12L:12D and a humidity of about 40%. The mice were housed individually in similar sized cages (41x26x15 cm) and were each provided with bedding (shredded paper and a square of blanket) and a toilet roll for cover. Food (rabbit pellets, Epol, JHB, RSA) and water were provided *ad libitum*. The mice were maintained under these conditions for two months while we awaited the arrival of the leptin.

5.3.1) *Acclimation:*

Prior to the arrival of the leptin, the two months were used as an acclimation period. Mice were weighed every second day and their reproductive status was assessed based on the position of the testes. Cages were cleaned on a weekly basis and all remaining uneaten food for that week was discarded. During the second last week of acclimation, the food provided to the animals was pre-weighed and at the end of the week all remaining uneaten food was collected and was used to calculate the weekly food intake for each mouse. This weekly food intake was then used to calculate a mean daily food intake, which was used in the experimental period.

5.3.2) *Experimental design:*

After the acclimation period, the 23 mice were separated into one of three groups such that the mean body mass and reproductive status of the groups were similar. Two of the groups each had eight mice and the third group consisted of seven individuals (Table 5.4). After dividing the mice into their experimental groups, they were all exposed to a reduced ambient temperature between

15 and 17^{o}C (photoperiod of 12L:12D and humidity of 40%) and a food-restricted diet. Mice were initially fed their daily food intake as calculated at 26^{o}C during acclimation. Since more energy will be expended in cold temperatures, this represents a form of food restriction. Thereafter food availability was manipulated to maintain a 10 to 25% reduction in mean body mass.

In addition to the changes in ambient temperature and food availability, mice were also subjected to one of three treatments. One group of eight mice received injections of exogenous leptin (40?g leptin in 100?l saline; dosage based on work by Ahima *et al.*, 1996 and Barash *et al.*, 1996), while the second group of eight received injections of saline only (100?l). The injections were administered subcutaneously, twice a day and 12 hours apart (between 06h00- 07h00 and 18h00-19h00). The third group of mice (n = 7) received no injections and are referred to as the control mice. The leptin was donated by Amgen (California, U.S.A.) and was a murine recombinant expressed in *E. coli*.

During the first week of the experimental period, five mice died (three from the leptin group; two from the control group) and some alterations were made to the experimental design. The temperature was increased from 15^{o}C to 17^{o}C, and food availability was increased and then slowly reduced over time. Two mice, matched in mass and reproductive status to two of the mice that had died within the leptin group, were moved from the saline group across to the leptin group. This new regime was continued for two weeks, after which the ambient temperature was decreased over two days to 15^{o}C. The experiment continued for another two weeks, at the end of which all the mice were sacrificed. In total, the experimental period lasted five weeks. Table 5.4 presents a summary of the experimental design including changes in mean body mass and changes in the number of mice per group.

For the first three weeks of the experimental period, mice were weighed every Monday, Wednesday and Friday, while for the last two weeks mice were weighed daily. The reproductive status of the mice was noted every second day and cages were cleaned once every week. Food was provided each morning after injecting and weighing the mice, and a record of the quantities of food provided for each mouse was kept. Once on food restriction, all mice consumed all the provided food, thus pair-feeding to control for any effect of leptin on appetite, was not necessary.

Table 5.4: Summary of the hormone manipulation period of the experiment to determine the effects of exogenous leptin on the reproductive activity of male *Rhabdomys pumilio*. Mean body masses and total number of mice per group are for the beginning of each week. Deaths are those that occurred during the week. Alterations are movements of mice to balance sample sizes.

Week	Temperature (°C)	Mean body mass ? 1SEM			Deaths by end of week	Alterations	Total number of mice/ group		
		control	saline	leptin			control	saline	leptin
1	15	41.6 ? 3.04	41.0 ? 2.66	41.4 ? 2.16	3 leptin 2 control	-	7	8	8
2	17	34.3 ? 1.50	34.6 ? 2.48	33.1 ? 1.80	-	2 from saline to leptin	5	6	7
3	17	35.3 ? 1.65	36.6 ? 2.30	33.6 ? 1.87	-	-	5	6	7
4	15	34.6 ? 1.64	36.6 ?1.85	33.9 ? 1.66	1 control, 2 leptin, 1 saline (dissected)	-	5	6	7
5	15	31.2 ? 1.98	31.9 ? 1.63	32.5 ? 2.09	All dissected	-	4	5	5

5.3.3) *Sacrifice, tissue collection, blood collection and histological analysis:*

At the end of the five weeks of food restriction the mice were sacrificed and processed as previously described. The reproductive organs were assessed as previously described, including the additional analyses of spermatogenesis (Chapter 3, 3.1.2 – 3.1.3, 3.5.3).

5.3.4) *Hormone assays:*

Plasma testosterone and plasma leptin were analysed as previously described (Chapter 3, 3.1.4 and 3.5.4, respectively).

5.3.5) *Statistical analysis:*

Statistical analyses were conducted using both SigmaStat and Statgraphics, as previously described.

5.4) Results:

While the first five mice that died were not used in the analyses, the four mice that died during the fourth week of the experiment were dissected and the data collected for these mice were included in the overall analyses of experimental data.

At the start of the experimental period, all of the males were scrotal except for one mouse in the saline group, which was partly-scrotal. The mean body masses of the three groups were not significantly different at the start of the experimental period, nor were they significantly different throughout the experimental period (week 1 to week 5). However, all the mice experienced a significant decrease in body mass from the start of the experimental period to the time of dissection ($P < 0.05$) such that they had lost between 20 and 25% of their original body mass. The reproductive status of the mice also changed during the experimental period and at the time of dissection three of the mice (one mouse from each group) were partly-scrotal, while all of the other mice were non-scrotal.

5.4.1) *Dissection data:*

At dissection, there was no significant difference in the mean body masses of the mice in the three groups ($P = 0.690$; Table 5.5) and all the mice had fat indices of zero. The mean masses of the testes, epididymides and accessory glands of the mice that received leptin were all significantly higher than those for the mice that received saline (Table 5.5), while the epididymides of the leptin group were also significantly heavier than those of the control group ($P < 0.05$).

Table 5.5: The effect of long-term treatment with leptin on body mass and on the masses of the reproductive organs of *Rhabdomys pumilio* recorded at sacrifice. All values are means ? 1 SEM.

	Mean masses (g)			P-value
	Control	**Saline**	**Leptin**	**(ANOVAs)**
Body	30.70? 2.05	32.79? 2.49	33.09? 1.53	0.690
Testes	0.44? 0.08^{a,b}	0.37? 0.05^{b}	0.60? 0.03^{a}	0.016
Epididymides	0.10? 0.02^{b}	0.07? 0.01^{b}	0.14? 0.01^{a}	0.007
Accessory glands	0.09? 0.02^{a,b}	0.06? 0.01^{b}	0.13? 0.02^{a}	0.048

- Within a row, groups with different superscript letters (a or b) are significantly different ($P < 0.05$).

5.4.2) *Histology:*

Histological analysis of the reproductive organs of the mice indicated that 16 of the 18 mice were reproductively active with more than 70% of the seminiferous tubules producing spermatozoa. The other two mice, one from the control group and the second from the saline group, had low spermatogenic activity (about 40%). One of these mice (saline treated) had no sperm in the epididymides and had low accessory gland activity (50%), while the second mouse (control) had some sperm in the epididymides (28% activity) and had fully active accessory glands (100%). Despite the low spermatogenic activities of these two mice, the mean activity of the testes did not differ significantly between the groups ($P = 0.552$; Table 5.6). There were no significant differences in the mean activities of the epididymides or accessory glands for the three groups ($P > 0.05$; Table 5.6) and the additional analyses on the seminiferous tubules yielded no significant differences between the groups (Table 5.7).

Table 5.6: The effect of long-term treatment with leptin on the activity of the reproductive organs collected from *Rhabdomys pumilio* at sacrifice. All values are means ? 1 SEM.

	Mean activity			**P-value (ANOVAs)**
	Control	**Saline**	**Leptin**	
Spermatogenic activity (testes)	77.2 ? 9.54	75.7 ? 8.16	88.4 ? 1.49	0.552
Sperm storage (epididymides)	69.6 ? 12.21	46.0 ? 13.61	80.3 ? 5.32	0.080
Accessory gland activity	100.0 ? 0.00	83.3 ? 10.54	92.9 ? 4.93	0.355

Table 5.7: The effects of long-term treatment with leptin on the number of spermatozoa produced by the seminiferous tubules of *Rhabdomys pumilio*, the number of germ cell layers forming the epithelial layer of the seminiferous tubules and the thickness of this layer. Values are means ? 1SEM.

	Mean values			**P-value (ANOVAs)**
	Control	**Saline**	**Leptin**	
Spermatozoa score	2.35 ? 0.31	2.15 ? 0.23	2.61 ? 0.19	0.412
Germ cell layers	3.52 ? 0.21	3.43 ? 0.19	3.63 ? 0.08	0.652
Thickness of layer (? m)	30.37 ? 1.77	30.27 ? 1.91	30.78 ? 0.85	0.966

5.4.3) *Hormone assays:*

Plasma testosterone levels ranged from <10ng/dL to 57ng/dL, with the control mice having the higher levels, and the saline mice having the lower levels (Table 5.8). The differences between the three groups were not significant (P = 0.482). Levels of plasma leptin-like immunoreactivity were higher in the leptin-treated mice than in the other three groups, however this difference was only significant between the leptin-treated and saline-treated mice (P = 0.009; Table 5.8).

Table 5.8: The effects of long-term treatment with leptin on the plasma testosterone and leptin-like immunoreactivity levels for *Rhabdomys pumilio* exposed to low ambient temperatures and reduced food availability. Values are means ? 1SEM.

	Mean values			**P-value (ANOVAs)**
	Control	**Saline**	**Leptin**	
Testosterone levels (ng/dL)	23.4 ? 11.41	12.7 ? 6.14	14.2 ? 4.97	0.482
Leptin levels (ng/ml)	8.20 ? 1.59^{a,b}	7.77 ? 1.13^{b}	26.00 ? 4.42^{a}	0.009

- within a row, groups with different superscript letters (a or b) are significantly different (P < 0.05).

5.5) Discussion:

The basic requirement for the short-term leptin experiment was that the mice were spermatogenically inactive at the start of the treatment period. Treatment with leptin for four days might then have been sufficient to initiate a new cycle of spermatogenesis through the stimulatory effect of leptin at the levels of the hypothalamus and anterior pituitary (Clarke & Henry, 1999; Foster & Nagatani, 1999; Moschos *et al.*, 2002) and this would have been detected via an increase in plasma LH levels. Although in the present experiment the mice were subjected to an energetic challenge, which has, in a previous study, inhibited spermatogenesis (Jackson & Bernard, 2001), spermatogenesis was not inhibited, and at dissection, all mice were spermatogenically active. Since spermatogenically active animals typically have elevated plasma LH levels (Williams-Ashman, 1988), it is not surprising that treatment with leptin did not have a significant effect on levels of plasma LH-like immunoreactivity. Although there were no significant differences in the levels of LH-like and leptin-like immunoreactivity, the mice treated with leptin had higher levels of LH-like immunoreactivity while the mice treated with saline had higher levels of leptin-like immunoreactivity. Previous experiments have reported an increase in LH levels when animals have been treated with leptin (Barash *et al.*, 1996; Yu *et al.*, 1997; Nagatani *et al.*, 1998; Cunningham *et al.*, 1999; Henson & Castracane, 2000) and it is possible that this increase is due to either leptin acting on the hypothalamus stimulating the release of GnRH (Clarke & Henry, 1999; Foster & Nagatani, 1999; Moschos *et al.*, 2002) and thereby an increase in LH production, or acting directly on the pituitary. Either or both of these options are plausible since receptors for leptin have been found in both the hypothalamus and the pituitary gland (McCann *et al*., 1998; Barkan *et al.*, 1999; Clarke & Henry, 1999, Tena-Sempere *et al*., 1999). The high levels of leptin-like immunoreactivity in the saline-treated mice is surprising, since they were not receiving exogenous leptin and had very little body fat. This might indicate a problem with the leptin assay although there is another possible explanation. It has been reported that testosterone suppresses leptin levels (see Holness *et al.*, 1999). Since the *Rhabdomys pumilio* that had received leptin had higher levels of LH-like immunoreactivity, it is likely that there would have been higher levels of testosterone in the blood of these mice and this could have suppressed leptin production.

In summary the short-term leptin experiment failed because the mice were spermatogenically active, and not inactive as required by the experimental design. It was not expected that treatment with leptin for four days would have had an effect on the mass and activity of the reproductive organs and indeed the results support this. However, the fact that the mice in the control group were spermatogenically active further reduced the likelihood of short-term treatment with leptin having any effect.

The main objective of the long-term experiment was to establish if treatment with exogenous leptin would counter the inhibitory effects of an energetic challenge on spermatogenesis. The key requirement for this experiment was that the mice were spermatogenically active at the start and although this was not confirmed by dissection, there is no reason to doubt it. The specimens were collected in late summer when they would normally have been spermatogenically active and were maintained under summer conditions with *ad libitum* food and water. At the start of the experiment all specimens but one were assessed as having scrotal testes. The energetic challenge used in this experiment was similar to that used previously (Jackson & Bernard, 2001) and it resulted in a significant decrease in body mass of all three groups. It is well established that treatment with leptin can cause a reduction in food intake (Ahima *et al*., 1996; Cunningham *et al.*, 1999; Figlewicz *et al.*, 2001) and a reduction in body weight (Barash *et al.*, 1996). Although it has been reported that long-term treatment with leptin will increase food consumption (Mounzih *et al.*, 1997), in the present experiment, all mice were on a food-restricted diet and consumed all the food that was provided, thus if treatment with leptin did suppress appetite, it did not suppress it to the extent that mice in this group left uneaten food. Furthermore, since there was no significant difference in the mean body mass of the three groups at the end of the experiment, the mass loss cannot be ascribed to treatment with leptin and probably simply reflects the effects of the energetic challenge.

The significant positive effect of leptin on the masses of the reproductive organs suggests that treatment with leptin did in some way override the inhibitory effects of the energetic challenge. Similar results have been observed in studies on both male and female mammals, where leptin treatment resulted in an increase in the weights of the reproductive organs (Barash *et al.*, 1996; Chehab *et al.*, 1997; Mounzih *et al.*, 1997). However, there were no significant effects of leptin on the levels of activity of the reproductive organs and this is contrary to what has previously been reported (Barash *et al.*, 1996; Almog *et al.*, 2001). This suggests that a first step in the inhibition of male reproductive activity is a reduction in the mass of the reproductive organs and that this is followed by a cessation of spermatogenesis and activity of the accessory glands. The masses of the reproductive organs of adult spermatogenically active mice in mid-summer average from 0.6g to 0.8g for the testes, 0.15g to 0.25g for the epididymides and 0.2g to 0.4g for the accessory glands (pers. obs.), and although these data cannot be compared statistically with those from the long-term experiment, it is clear that they are substantially higher than those of the control and saline groups but very similar to those of the leptin-treated mice (Table 5.9). Since the mice for the long-term experiment were caught in late summer and were maintained under summer conditions, it is likely that at the start of the treatment period (energetic challenge and leptin or saline injections) the reproductive organs were similar in size to the measurements given for the spermatogenically active mice (mid-summer, Table 5.9). This further supports the suggestion that the first effect of an energetic challenge is a reduction in mass that occurs without any apparent change in activity, and that this can be overridden by exogenous leptin.

A previous study has shown that a long-term (four weeks) energetic challenge will inhibit spermatogenesis in *Rhabdomys pumilio* (Jackson & Bernard, 2001) and there are two possible explanations for why a similar inhibition did not occur in this study. Firstly, it is possible that the mice used in the present study had larger fat reserves than the mice used previously, and these fat reserves were used to maintain spermatogenesis. The two years that preceded this experiment were particularly mild (see Chapter 3) and it is possible that the mice were in a better condition than those used in the previous study of Jackson and Bernard (2001). Secondly, it is possible that the present study was not continued for long enough to detect an inhibition of spermatogenesis.

Table 5.9: Comparison of the mean masses of the reproductive organs of spermatogenically active, male *Rhabdomys pumilio* collected in mid-summer, and of males from the long-term leptin experiment. Values are means; standard errors have been excluded.

		Long-term leptin experiment		
	Mid-summer	**Control**	**Saline**	**Leptin**
Testes (g)	0.60 – 0.80	0.44	0.37	0.60
Epididymides (g)	0.15 – 0.25	0.10	0.07	0.14
Acc. gld. (g)	0.20 – 0.40	0.09	0.06	0.13

Since all specimens were spermatogenically active, it is not surprising that there was no significant difference in plasma testosterone levels. Plasma testosterone levels were not measured in the first experiment because the short duration reduced the likelihood of triggering a measurable increase in testosterone production.

In summary, while the short-term experiment suggested the possibility that exogenous leptin stimulated LH production, the fact that the energetic challenge had not inhibited spermatogenesis made the results difficult to interpret. The long-term experiment produced more significant results with exogenous leptin reducing mass loss of the reproductive organs. It appears that the first response of male *Rhabdomys pumilio* to a prolonged energetic challenge is a reduction in the size of the reproductive organs, that occurs without a significant change in plasma testosterone levels or spermatogenic activity.

CHAPTER SIX: FAT METABOLISM AND ITS EFFECTS ON REPRODUCTIVE ACTIVITY IN MALE *RHABDOMYS PUMILIO*.

In a previous study that examined the effects of an energetic challenge (reduced food availability and reduced ambient temperature) on spermatogenesis of *Rhabdomys pumilio*, it was noted that certain mice had remained reproductively active despite the energetic challenge and that these mice had maintained body fat stores (Jackson & Bernard, 2001). Since a reduction in food should result in a decrease in body fat (Bronson *et al.*, 1991), the results from this study were unexpected and pointed to a link between body fat stores and reproductive activity in *Rhabdomys pumilio*. Mice that were able to maintain a fat store in the face of an energetic challenge had the potential to remain reproductively active. Such a link has been demonstrated in other mammals, including squirrels, where reductions in body fat were associated with delayed reproductive development (Forger *et al.*, 1986), and hamsters, where heavier and fatter females either prolonged or maintained their oestrous cycles despite food deprivation (Schneider & Wade, 1989, 1990a, 1990b). Since is has been shown that body mass alone does not influence ovulation in rats (Trujillo & Broughton, 1995), it is evident that fat reserves are the more likely factor influencing reproductive activity.

Fat stores serve a variety of functions in the vertebrate body, the main one being to act as a caloric reserve providing a protective barrier against the climatic challenges of winter and possible food deprivation (Ponds, 1978 – cited by Bronson, 1987; Bronson *et al.*, 1991; Tropp & Markus, 2001). Fat also affects reproductive activity and regulates the occurrence of ovulation (Frisch, 1984; Frisch 1987- cited by Bronson *et al.*, 1991), gonadotropin secretion (Frisch, 1984; Bronson *et al.*, 1991), alters ovarian follicle and corpus luteum functioning (Mattos *et al.*, 2000), and increases the precursors available for the synthesis of reproductive hormones (Frisch, 1984; Mattos *et al.*, 2000). Fat may affect reproduction via one or both of two mechanisms. Firstly, fat acts as a signal to the reproductive axis indicating when energy reserves are sufficient for reproduction (Frisch, 1984; Forger *et al.*, 1986; Schneider & Wade, 1989). Recently it has been discovered that white adipose tissue produces the hormone leptin and it is believed that this hormone is the signal linking fat stores to the endocrine system and thus controlling reproduction (see Chapter 5). Secondly, it is theorised that critical levels of fat are required before puberty and other reproductive processes will occur, although this function of fat is still being debated (Frisch, 1984; Frisch 1987- cited by Bronson *et al.*, 1991; Foster & Nagatani, 1999).

Although it has been suggested that fat stores are too labile to effectively co-ordinate puberty with somatic development (Bronson *et al.*, 1991), it is also thought that because fat stores are labile, they will reflect environmental changes in food supplies more rapidly than other tissues (Frisch, 1984). Hence, a signal that emanates from the fat store could provide a means by which reproduction is modified in response to a change in the environment. Since the discovery of leptin, many researchers have concentrated on establishing its role in the control of reproduction (see Chapter 5) however, research into the role of fat as a caloric reserve is ongoing, including investigations in to how fat metabolism itself can affect reproductive activity. By blocking the metabolism of fat using either methyl palmoxirate (MP) or mercaptoacetate (MA), researchers (e.g. Schneider & Wade, 1989, 1990b; Dark *et al.*, 1994) have shown that a disruption in fat metabolism, when combined with secondary factors such as reduced food availability, can negatively affect the oestrous cycle (Schneider & Wade, 1989).

It is therefore evident that body fat may affect reproductive activity via two quite different mechanisms, firstly by functioning as a caloric reserve and secondly by acting as an endocrine gland. Although leptin production and fat metabolism itself are probably linked, the focus of this chapter is to investigate the effects of fat metabolism on reproductive activity by determining whether a high fat diet and/or treatment with mercaptoacetate will affect spermatogenesis in *Rhabdomys pumilio*. It was hypothesised that treatment of obese mice with mercaptoacetate, while exposed to an energetic challenge, would block metabolism of their fat stores and cause a more rapid inhibition of spermatogenesis than in mice that were provided with a high fat diet while exposed to the same energetic challenge.

6.1) Materials & Methods:

Adult, male *Rhabdomys pumilio* were trapped in Thomas Baines Nature Reserve over a two-week period during March 2001 (late summer). These mice were brought into the laboratory and placed individually into similar sized cages (41x26x15 cm) in a CE room set at 26°C, with a photoperiod of 12L:12D, and a humidity of about 40%. Mice were provided with shredded paper and a square of blanket for bedding, a toilet roll for cover, and food (Rabbit pellets, EPOL, JHB, R.S.A.) and water were provided *ad libitum*.

6.1.1) *Initial fattening stage:*

Once 33 mice had been caught, they were fed a mixed diet of normal rabbit pellets (normal diet) and rabbit pellets that had been soaked in vegetable fat (Holsum; high fat diet), alternating the two diets every second day. This feeding regime, which was used to encourage an increase in body weight and body fat reserves, continued for 14 weeks, by the end of which all the mice were above 40g in mass and considered obese. Throughout this period food and water were provided *ad libitum* and cages were cleaned once a week. During the last week of the period of fattening, the food was weighed before being given to each mouse and two days later, when the diet was changed, the remaining uneaten food for the two days was collected and used to calculate a mean daily food intake for that diet type. Throughout the 14 weeks, the mice were weighed and their reproductive status assessed (according to the position of the testes within the scrotal sacs) every second day.

6.1.2) *Treatments:*

At the end of the period of fattening, the mice were divided into four groups such that there was no significant difference in the mean body mass or reproductive condition (as assessed externally) between the groups. Nine mice were allocated to the *ad libitum* group and were maintained on an *ad libitum* diet (alternating the diet type every second day) for the remainder of the experiment. This group received no injections and in the remainder of this chapter are labelled the AL group. The remaining mice were all exposed to a food-restricted diet and received their daily food intake less 10% (changing diet type every two days). Within this food restricted group, mice were divided equally (n = 8) into three groups: a food-restricted group with no other treatment (FR), a food-restricted group in which the mice received daily injections of 200?l saline (FRS), and a food-restricted group in which the mice received daily injections of mercaptoacetate (MA - Sigma, St. Louis, U.S.A; 600?mol/kg body mass) (FRMA). All injections were given at 07h30 and were administered subcutaneously.

This treatment period lasted for four weeks and was conducted in a CE room set at a reduced temperature of 15°C (typical winter temperature) with a photoperiod of 12L:12D and humidity of about 40%. Every second day, prior to receiving food, the mice were weighed and their reproductive status was recorded. If the body mass of a mouse decreased below 70% of its mean mass as calculated during the feeding period (6.1.1), the food provided to the individual was increased to allow a slight increase in body mass to above the 70% level.

6.1.3) *Sacrifice, tissue collection and blood collection:*

On the day following the last treatment, the mice were sacrificed by injection with 0.2ml Euthanaze. The mice were then weighed and the testes, epididymides and accessory glands were removed, weighed to the nearest 0.001g and placed into Bouin's fixative. Blood was collected from the heart by ventricular puncture and processed as described in Chapter 3 (3.1.2). A subjective fat index was determined (Jackson & Bernard, 2001).

6.1.4) *Histological analysis and hormone assays:*

Reproductive organs were processed and assessed as previously described (Chapter 3: 3.1.3 and 3.1.3.1). Blood was assayed for testosterone and leptin as previously described (Chapter 3: 3.1.4 and 3.4.5, respectively).

6.1.5) *Statistical analysis:*

Statistical analyses were conducted using both SigmaStat and Statgraphics. Individuals were compared with Student's t-tests, while comparisons between the experimental groups were conducted using analyses of variance (ANOVA) or the non-parametric equivalents where applicable. Percentages were arcsine transformed for statistical analysis however these data are presented as percentages within figures and tables. Differences were considered significant at a < 0.05 and values are expressed as means ± 1 SEM, unless otherwise stated.

6.2) Results:

Of the 33 mice caught in the wild, 11 were non-scrotal, 10 were partly-scrotal and 12 scrotal. Once in the laboratory, the mice were fed *ad libitum* for 14 weeks during which their body masses increased (Figure 6.1) and their reproductive status changed so that 24 mice were scrotal, seven were partly-scrotal and two were non-scrotal. At the start of the treatment period, the mice were divided into four groups such that the mean body mass of each group of mice was not significantly different ($P = 0.989$) and the reproductive status of members of the groups was similar (Table 6.1).

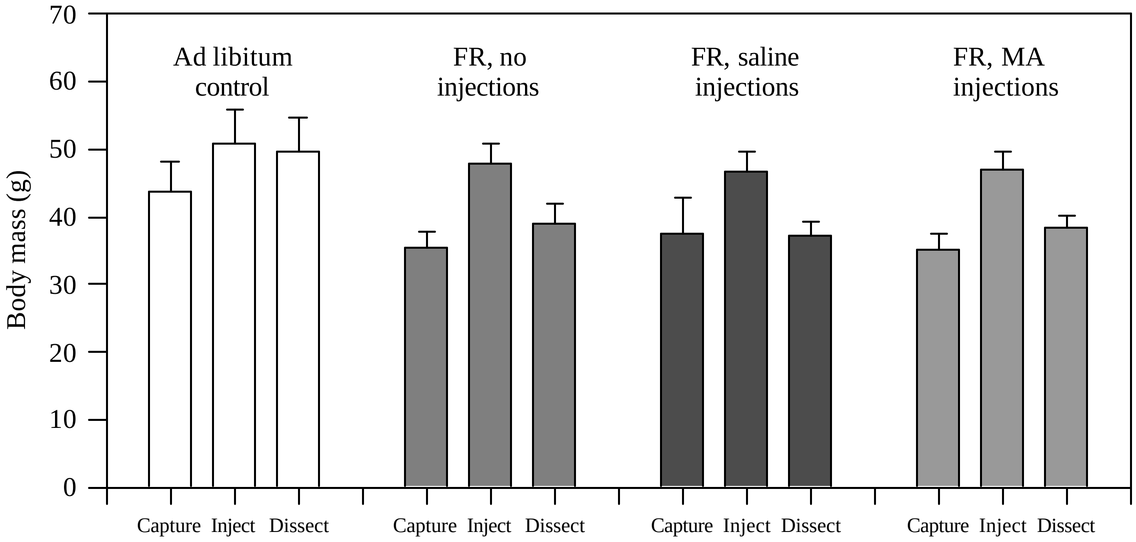

Figure 6.1: Mean body mass of the four groups of mice at capture (Capture), at the start of the treatment period (Inject) and at dissection (Dissect.). FR = food restricted, MA = mercaptoacetate. Means are ? 1SEM.

Table 6.1: The reproductive status of mice in the four experimental groups at the start of the treatment period.

	Number of mice		
Group*	**Non-scrotal**	**Partly-scrotal**	**Scrotal**
AL	0	3	6
FR	0	1	7
FRS	1	1	6
FRMA	1	2	5

*AL = ad libitum diet; FR = food restricted, no injections; FRS = food restricted, saline injections; FRMA = food restricted, mercaptoacetate injections.

During the first week of treatment, three mice within the food-restricted groups died and some alterations were made to compensate for these losses. Two mice were removed from the AL group (revised sample size = 7) and of these, one was placed into the FR group in which there had been two deaths (revised sample size = 7) and the second mouse was placed into the FRMA group in which there had been one death (revised sample size = 8). During the remaining weeks of the treatment period another four mice died and final sample sizes for the four groups at dissection were: AL = 7; FR = 6; FRS = 6; FRMA = 7.

There was a statistically significant decrease in the mean body mass, from the start of the treatment period to dissection, within each of the three groups of mice on the food-restricted diet ($P < 0.01$ for all; Figure 6.1; Table 6.2). The mean body mass of the AL group decreased, but not significantly, during the treatment period (Figure 6.1, Table 6.2). At dissection, the mean body masses of the mice in the food-restricted groups were all significantly lower than the mean body mass of the mice in the AL group ($P = 0.045$), although there was no significant difference between the mean body masses of the mice in the three food-restricted groups (Figure 6.1; Table 6.2).

Table 6.2: Mean body masses of the four groups of mice at the start of the treatment period and at dissection, indicating weight changes during the experiment within a group (t-tests) and differences between the groups (ANOVAs). Values are means ? 1SEM.

Group*	Mean body mass at start of treatment	Mean body mass at dissection	P-value (t-tests)
AL	50.7 ± 5.03	49.8 ± 4.94^{a}	0.318
FR	47.8 ± 2.97	38.9 ± 3.13^{b}	0.002
FRS	46.6 ± 3.15	37.1 ± 2.26^{b}	0.005
FRMA	47.0 ± 2.71	38.3 ± 1.93^{b}	0.003
P-value (ANOVAs)	0.989	0.045	

*AL = ad libitum diet; FR = food restricted, no injections; FRS = food restricted, saline injections; FRMA = food restricted, mercaptoacetate injections.

- Within a column, groups with different superscript letters (a or b) are significantly different ($P < 0.05$).

The reproductive status of the mice also changed during the treatment period and at dissection all of the mice in the food-restricted groups were either partly-scrotal or non-scrotal, while mice in the AL group were either scrotal, partly-scrotal or non-scrotal (Table 6.3). There was a significant decrease in the number of scrotal mice from the start of the treatment period to dissection within the three food-restricted groups (P < 0.001).

Table 6.3: The reproductive status of mice (with percentages in parentheses) within each of the four groups as assessed at dissection. (Data from Table 6.1 indicating the reproductive status of mice at the start of the treatment period have been included to allow for easy comparison.)

		Number of mice		
Group*		**non-scrotal**	**partly-scrotal**	**scrotal**
AL	**START**	0	3 (33%)	6 (67%)
	Dissection	3 (43%)	1 (14%)	3 (43%)
FR	**Start**	0	1 (12.5%)	7 (87.5%)
	Dissection	4 (67%)	2 (33%)	0
FRS	**Start**	1 (12.5%)	1 (12.5%)	6 (75%)
	Dissection	3 (50%)	3 (50%)	0
FRMA	**Start**	1 (12.5%)	2 (25%)	5 (62.5%)
	Dissection	4 (57%)	3 (43%)	0

*AL = ad libitum diet; FR = food restricted, no injections; FRS = food restricted, saline injections; FRMA = food restricted, mercaptoacetate injections.

6.2.1) *Dissection:*

The mean masses of the testes, epididymides and accessory glands of the mice in the food-restricted groups were lighter than those of mice in the AL group (Table 6.4). Statistical analysis of these data indicated that the three groups of mice on the food-restricted diet had significantly lighter accessory glands than the mice in the AL group (P = 0.005); the mice receiving injections of saline or mercaptoacetate (FRS and FRMA) had significantly lighter epididymides than the mice in the AL group (P = 0.036); while there was no significant difference in the mean masses of the testes (P = 0.130) of the mice in the four groups (Table 6.4). Mice in the AL group had significantly more body fat than the mice from the food-restricted groups receiving injections (FRS and FRMA; P = 0.008; Table 6.5) while there was no significant difference in mean fat scores between the mice from the three food-restricted groups (Table 6.5).

Table 6.4: Mean masses of the reproductive organs for mice at dissection after the fat metabolism experiment. P-values are from ANOVAs. Values are means ? 1SEM.

Organs	*AL mice	*FR mice	*FRS mice	*FRMA mice	P-value
Testes (g)	0.84 ± 0.07	0.67 ± 0.12	0.52 ± 0.09	0.66 ± 0.09	0.130
Epididymides (g)	0.27 ± 0.03^{a}	0.19 ± 0.04^{a,b}	0.15 ± 0.03^{b}	0.17 ± 0.02^{b}	0.036
Accessory glands (g)	0.60 ± 0.09^{a}	0.29 ± 0.08^{b}	0.21 ± 0.06^{b}	0.24 ± 0.07^{b}	0.005

*AL = ad libitum diet; FR = food restricted, no injections; FRS = food restricted, saline injections; FRMA = food restricted, mercaptoacetate injections.

-Within a row, groups with different superscript letters (a or b) are significantly different ($P < 0.05$).

Table 6.5: The number of mice from each treatment group, in each of the fat score categories after the fat metabolism experiment.

Group	Fat score				Mean fat score
	0	**1**	**2**	**3**	
AL	1	2	2	2	1.17 ? 0.42^{a}
FR	4	2	0	0	0.33 ? 0.21^{a,b}
FRS	5	1	0	0	0.17 ? 0.17^{b}
FRMA	6	1	0	0	0.14 ? 0.14^{b}
				P-value =	0.008

*AL = ad libitum diet; FR = food restricted, no injections; FRS = food restricted, saline injections; FRMA = food restricted, mercaptoacetate injections.

- Within column, groups with different superscript letters (a or b) are significantly different ($P < 0.05$).

6.2.2) *Histology:*

All the mice were spermatogenically active, with sperm stored in the epididymides and secretory material in the accessory glands. Compared to the mice in the AL group, the three groups of mice on the food-restricted diet had lower levels of reproductive organ activity (Figure 6.2), however none of these differences were statistically significant.

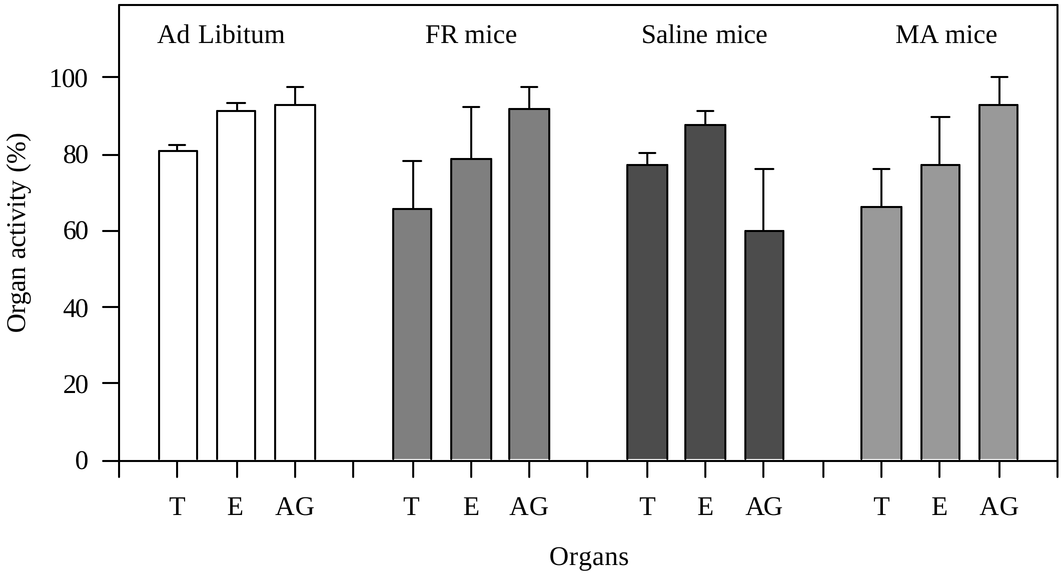

Figure 6.2: Mean activities of the reproductive organs at dissection. T = testes; E = epididymides; AG = accessory glands. FR = food-restricted. MA = mercaptoacetate. Means are ? 1SEM.

6.2.3) *Hormone assays:*

The mean plasma testosterone level of the mice in the AL group was significantly higher than levels measured in the plasma of mice in the three food-restricted groups (P = 0.035). The FR group had the lowest testosterone levels of all groups, while the FRS group had the highest levels for the food-restricted groups (Table 6.6). The differences between the mean plasma testosterone levels for the mice in the three food-restricted groups were not statistically significant (P>0.05). The mean levels of plasma leptin-like immunoreactivity of the four groups of mice were not significantly different from each other (Table 6.6).

Table 6.6: Plasma testosterone and levels of leptin-like immunoreactivity for the four treatment groups at dissection. Values are means ? 1SEM.

	Mean values				**P-value**
	AL	**FR**	**FRS**	**FRMA**	**(ANOVAs)**
Testosterone (ng/dL)	64.6? 15.16^{a}	19.6 ? 6.45^{b}	33.1? 8.38^{b}	30.9 ? 6.69^{b}	0.035
Leptin (ng/dL)	5.78 1.24	5.98 1.43	6.50 1.47	5.80 0.93	0.979

*AL = ad libitum diet; FR = food restricted, no injections; FRS = food restricted, saline injections; FRMA = food restricted, mercaptoacetate injections.

-Within a row, groups with different superscript letters (a or b) are significantly different ($P < 0.05$).

6.3) Discussion:

The experimental design required the mice to be spermatogenically active with large fat stores at the start of the treatment period. It was not possible to confirm either of these conditions without sacrificing specimens, but 14 weeks on the high fat diet had resulted in an increase in body mass and an increase in the number of mice with scrotal testes. Similar improvements in reproduction have been recorded in ruminants when fat was included in their diets (see Mattos *et al*., 2000).

The mice were then exposed to an energetic challenge (reduced ambient temperature and reduced food availability) that was expected to result in loss of body mass and body fat reserves and a reduction in reproductive activity. Indeed, this appears to have occurred and while there was no significant change in the body mass of the mice that continued to receive the *ad libitum* diet, the mean body mass of those mice on the food restriction diets decreased significantly. In addition, food restriction resulted in a significant loss of body fat, reduction in the number of mice with scrotal testes, reduction in mass of the accessory glands and plasma testosterone levels. However, treatment with mercaptoacetate was expected to prevent fat metabolism and thus exaggerate the effects of the energetic challenge and this did not happen. Daily injections, with either saline or MA, had no significant effect on body mass, fat score, mass of the testes and accessory glands or plasma testosterone levels. The only significant effect of these injections was to reduce the mass of the epididymides and it is hard to conceive of a biological explanation for this. In spite of the changes in body size, the size of the reproductive organs and plasma testosterone levels, neither the energetic challenge nor treatment with MA had a significant inhibitory effect on spermatogenesis, as assessed histologically.

The fact that MA appears to have had no overall effect on the treated mice was surprising, especially on the metabolism of fat, since no fat was recorded in these mice at dissection. A possible explanation for this may be that the dosage of MA was too low. Dark *et al.* (1994) experimented with a number of dosages for MA, ranging from 400 to 1200 ? mol/kg body mass, to test the effects of the disruption of fatty acid oxidation on the torpor of *Phodopus sungorus*. Although MA only affected the torpor of these hamsters when administered in combination with 2-deoxy-d-glucose (2DG), Dark *et al.* (1994) found that 800? mol/kg was the optimal dose, which caused a reduction in whole blood glucose concentrations and an increase in food intake. However, for the current experiment, 600? mol/kg was used since 800? mol/kg is considered to be too high and may produce signs of toxicity (S. Ritter – Washington State University, pers. comm.). It is also possible that the mice did not in fact develop fat stores during the 14-week fattening period. However this is unlikely since the mice received a high fat diet for a prolonged period during which they were exposed to a high ambient temperature (26°C). Furthermore, the fact that the mice on the AL diet had a significantly higher mean body fat score at dissection than the mice on the food restricted diets suggests that all mice had developed fat reserves during the fattening period.

The fact that the two groups of mice that were exposed to food restriction only and food restriction with saline injections remained spermatogenically active despite the energetic challenge may be due to two factors. Firstly, the animals would have been able to metabolise their fat stores and thus delay reproductive inhibition. Secondly, the additional energy provided by the fat in the diet may have been sufficient to balance the costs imposed by the energetic challenge. Previous studies on other mammals have indicated that such factors can affect reproduction whereby fatter animals can maintain reproductive activity despite an energetic challenge (Schneider & Wade, 1989, 1990a) and that diets with supplemented fat can delay the effects of food deprivation (Tropp & Markus, 2001) and improve reproductive activity (Schneider & Wade, 1990a; Mattos *et al.*, 2000).

To return to the mice treated with MA, the most parsimonious explanation for the results is that MA failed to inhibit fatty acid metabolism. This could have been related to the dose used or to some other reason, but in the absence of an inhibition of fatty acid metabolism, these mice responded in the same way as the other mice on the food-restricted diet. It should however be noted that animals can compensate for an MA-induced energy deficit. In an experiment to determine the effects of MA on macro-nutrient intake, Ritter *et al.* (1999) found that rats increased their consumption of proteins and it was suggested that the MA-induced decrease in the availability of fat for energy metabolism might increase the utilisation of other substrates. This alteration in use of energy stores has been confirmed by other studies where animals treated with methyl palmoxirate (MP) and 2-DG, which prevent oxidation of fatty acids and glucose respectively, resulted in an inhibition of reproductive activity (Schneider & Wade, 1989; Dark *et al.*, 1994).

In spite of the apparent failure of this experiment, the results do provide some further insight into the control of reproduction in *Rhabdomys pumilio*. The energetic challenge resulted in a reduction in body mass, fat reserves and plasma testosterone levels, pointing once again to a link between fat reserves and reproduction. Development of the reproductive accessory glands is stimulated by testosterone (Coffey, 1998) and these glands were the most sensitive to the energetic challenge. It is possible that the duration of the energetic challenge was too short and that a longer period would have resulted in a greater effect on spermatogenesis. However the very nature of these experiments, which put the mice under stress, makes it difficult to balance a prolonged experiment with survival of the animals.

The continuation of spermatogenesis in the face of a substantial energetic challenge may be an integral part of an opportunistic reproductive strategy and will be addressed in the general discussion (Chapter 7).

CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSIONS

The broad aim of this study was to use field and laboratory experiments to examine the way in which the environment and the endocrine system combine to control reproduction in a small mammal from a seasonal but unpredictable environment. *Rhabdomys pumilio* was selected as the model species because it is common in the Eastern Cape Province, reproduces seasonally in the field (although there are a few exceptions, David & Jarvis, 1985) and reproduces under laboratory conditions. Previous studies have shown that day length alone does not inhibit spermatogenesis (Jackson & Bernard, 1999), a reduction in food availability and low ambient temperature (i.e. an energetic challenge) result in an inhibition of spermatogenesis in male *Rhabdomys pumilio*, and that females are more susceptible to inhibition than males (Jackson & Bernard, 2001). The Eastern Cape Province was used as the study area because it is characterised by a seasonal but unpredictable climate. The results of the individual experiments have been discussed in the earlier chapters and the aim of this final chapter is to present an overall synthesis of the key findings.

The results of all the experiments indicated that an energetic challenge, either natural, as in winter at Thomas Baines Nature Reserve and MZNP, or laboratory based, in terms of a reduction in food quantity and ambient temperature, resulted in a reduction in the body mass of the animals, a reduction in the masses of the reproductive organs, and, in the field experiment at MZNP, a reduction in the number of pregnant females. At the end of the field and laboratory experiments the animals had no body fat stores. This may indicate that the energetic challenges caused a loss of body fat, and while this is the most likely explanation, it could not be confirmed, as the body fat content at the start of the experiments was unknown. In spite of the often significant reduction in mass of the reproductive organs, the field and laboratory energetic challenges did not inhibit spermatogenesis. This was surprising, since the laboratory conditions used in the current study were similar to those used by Jackson & Bernard (2001) which did inhibit spermatogenesis. It is possible that the energetic challenges were not continued for long enough in the present experiments to inhibit spermatogenesis, but this is unlikely since the present experiments all ran for between 21 days and 28 days and Bernard & Jackson (2001) reported inhibition of spermatogenesis after 28 days. It is also possible that the mice used in the present experiments were in better condition, with greater body fat stores than those used by Bernard & Jackson (2001). 2000 and 2001 were particularly mild years in the Eastern Cape Province (see Chapter 2) and this may have resulted in a widespread increase in general body condition which could have allowed *Rhabdomys pumilio* to counter the energetic challenges. In the experiment that looked at the effect of inhibiting fat metabolism (Chapter 6), mice were fed a high fat diet and this could have provided the necessary energy to maintain spermatogenesis. It is worth noting that while the inhibition of reproduction achieved by Jackson & Bernard (2001) was based on a single experiment, the results presented here are based on three field and three laboratory experiments over three years and should therefore be regarded as the norm.

Although the responses to the natural (field) and laboratory energetic challenges were not as marked as expected, the results of the experiments clearly indicate that the effects of an energetic challenge can be countered. Firstly, in the first two field food supplementation experiments at Thomas Baines Nature Reserve, winter was particularly mild and both male and female mice remained reproductively active with high numbers of pregnant females and juveniles being present on both supplemented and control grids. This suggests that under natural conditions, a mild winter, with associated increased food availability and reduced thermoregulatory costs, can promote opportunistic reproduction in *Rhabdomys pumilio*. In contrast, in MZNP, where the winter was typically harsh and dry, no pregnant females or juveniles were present on the study site after May (the end of the breeding season) although males on both grids were spermatogenically active. Food supplementation at MZNP reduced the effects of this natural energetic challenge and mice that received supplementary food were larger, more reproductively active (as assessed externally) and had larger reproductive organs than the mice on the control grid. However, the provision of supplementary food did not result in pregnancy. The results from the three field experiments suggest that the normal winter inhibition of reproduction in the Eastern Cape Province (Perrin, 1980) can be completely overridden by one or more unusually mild winters (as seen at Thomas Baines Nature Reserve) or partially overridden by the provision of supplementary food (as seen at MZNP). The results further support the idea that females are more susceptible to environmental inhibition of reproduction than males (Pryor & Bronson, 1981; Bronner *et al.*, 1988; Wade *et al.*, 1996; Tilbrook *et al.*, 2000; Jackson & Bernard, 2001). The results from the MZNP experiment are supported by those from the MA experiment (Chapter 6), in which the provision of a high fat diet countered the effects of reduced ambient temperature and food availability.

The nature of the link between the environmental cues and the HPG axis was explored in the three laboratory based experiments. Provision of exogenous GnRH to *Rhabdomys pumilio* that had been exposed to a prolonged energetic challenge resulted in a significant increase in the masses of the testes and epididymides, and an increase in the activity of the reproductive organs. Similarly, provision of exogenous leptin concurrently with an energetic challenge countered the negative effects of the energetic challenge, so that the treated males had larger reproductive organs. The attempt to inhibit fat metabolism with MA was unsuccessful.

Combining the results from the field and laboratory experiments, it appears that the normal winter inhibition of reproduction can be overridden if the energetic challenge in winter is reduced either naturally (Thomas Baines Nature Reserve 2000 & 2001) or through provision of supplementary food (MZNP 2002). Furthermore, females are more sensitive to an energetic challenge than are males and reproduction is more readily inhibited. Since exogenous leptin and GnRH will counter the effects of an energetic challenge, it is likely that the link between the environmental cues and the endocrine system is the white fat store. Thus one could speculate that, in response to an energetic challenge, the fat store is depleted, leptin levels decline and this negatively affects the pulsatile release of GnRH. In response to this, LH and FSH levels will decrease (Perheentupa *et al.*, 1995; Garrel *et al.*, 1998; Raga *et al.*, 1999; Marshall *et al.* 2001; although not seen in the present study), and this will bring about a decrease in the size of the reproductive organs and levels of gonadal steroid hormones. In the females, the reduction in organ mass is associated with a cessation of reproductive activity while in males it is not and spermatogenesis continues. In the present study, the failure of the natural and artificial energetic challenges to inhibit spermatogenesis may be explained in terms of the same neuroendocrine pathway and any endogenous or exogenous source of leptin or GnRH would be expected to override the energetic challenge. For example, access to a high fat diet, similar to that used in the MA experiment, could result in the maintenance of a fat store and thus elevated leptin levels. Or, there might be a natural exogenous source of a GnRH-like compound in the field, such as oats, which has been found to release a peptide similar to GnRH and thus affect reproductive activity (I'Anson *et al.*, 1991).

The results of this study support the suggestion that reproduction in *Rhabdomys pumilio* is opportunistic in the truest sense (Jackson & Bernard, 2001), with males remaining spermatogenically active at all times of the year (if beneficial to do so). Females are more sensitive to reproductive inhibition and control the timing of reproduction, which may be seasonal or continuous, depending on the availability of energy stores (Bronson & Perrigo, 1987; Bronson & Heideman, 1994). Such opportunistic reproduction is ideally suited to an unpredictable environment since it allows a rapid response to a change in the environment;

successful reproduction may occur in winter if conditions are suitable and reproduction may be inhibited in summer if there is an unusual energetic challenge. This response need not be population-wide but will occur at an individual level and this explains why there are reported instances of reproductively active *Rhabdomys pumilio* in winter (David & Jarvis, 1985; Jackson & Bernard, 2001).

Finally, while this study has concentrated on the role of an energetic challenge, it is possible that other environmental variables and endocrine pathways may be involved in the control of reproduction in *Rhabdomys pumilio*. Further studies on the control of reproduction of *Rhabdomys pumilio* should focus on the females, since it appears that they are more sensitive to reproductive inhibition than the males.

APPENDIX

Mallory's trichrome stain (Humason, 1967):

Solution:

Distilled water	200.0ml
Dissolve each of below before adding next stain:	
Phosphotungstic acid	1.0g
Orange G, C.I. 16230	2.0g
Aniline blue, WS, C.I. 42780	1.0g
Acid fuchsin, C.I. 42685	3.0g

Procedure:

Hydrate slides to water – 5mins per stage from xylene to water.

Stain for 5min and then wash in running water for ½ a minute.

Dehydrate slides – 5mins per stage from water to xylene

Mount slides and allow them to dry.

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