IMPACT OF 24H LIGHT ON PHYSIOLOGY OF TRANSGENIC GH MICE

AN INTEGRATIVE STUDY OF REPRODUCTION, FEEDING AND BEHAVIOURAL ACTIVITY IN GIANT TRANSGENIC GROWTH HORMONE MICE

By

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ABSTRACT

"Supermice" (TRrGH mice) contain multiple copies of rat growth hormone genes incorporated into a single chromosome. This results in double normal growth rates reaching adult body sizes twice that of normal mice. To determine how exposure to constant light (LL) affects various physiological processes, reproduction, feeding, and behaviour were examined in LL-reared TRrGH mice. Fertility, organ allometries, feeding rates, behavioural time budgets, and circadian feeding and sleep rhythms were compared for both LL and standard 12h dark:12h light (LD).

Both TRrGH and normal females exhibited a significant decrease in fertility in LL. On a mass-specific basis, TRrGH females showed increased combined ovary mass and a reduction in thymus and heart size in LL. TRrGH males demonstrated increased testes mass in LL. When adrenal size was compared between males and females, both TRrGH and normal females exhibited larger adrenals than their male counterparts in both light treatments.

The fertility decrease observed in LL may have been associated with reduced food intake. LL-reared TRrGH females ate less than those in LD, although significantly more than TRrGH males in both LL and LD. When compared to normal mice, both sexes of TRrGH mice ate less in both photoperiods. The feeding rates of transgenic GLUT-4 mice were also examined. GLUT-4 mice contain double the amount of insulin responsive GLUT-4 glucose transporters which results in an increased blood glucose clearance rate. These mice, like TRrGH mice, ate less than normals, although a different age-related feeding pattern was observed.

TRrGH mice in LL are behaviourally more lethargic than those reared in LD, and spend less time feeding and drinking. Circadian feeding and sleep patterns were shifted in LL by approximately 12 hours, and exhibited reduced peak amplitudes. Ultradian patterns appeared to survive the breakdown of circadian organization.

TRrGH mice demonstrate a hormonal imbalance due to the excess allocation of energy into growth. It appears that, in LL, hormonal systems are further altered resulting in an increase in reproductive impairment assiciated with reduced feeding. One of these altered hormones may be estrogen. Hormones involved in hypothalamic-pituitary-adrenal axis (stress axis) are also implicated. It is concluded that photoperiod is important in regulating physiological processes, and TRrGH mice are more susceptible to environmental alterations due to their altered endocrinological state.

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PREFACE

The primary goal of our Supermouse (TRrGH mouse) research programme was to extend knowledge concerning the physiological and behavioural implications of genetically engineered growth enhancement in vertebrates. The emphasis of the present study centers on the altered physiological resource allocation in constant light (LL) and the associated impacts on fertility of mice engineered for elevated growth via the incorporation of multiple copies of rat growth hormone fusion genes.

The *General Introduction* examines the basic concepts underlying the infertility observed in transgenic growth hormone mice, as well as the "principle of allocation", a fundamental tenet of life-history theory. Descriptions of gene transfer techniques provides essential background regarding the incorporation and expression of foreign gene constructs. A basic description of reproductiove endocrinology is also provided.

Work described in this thesis is presented in the form of three articles. Each is preceeded by a brief description of the experimental approach, as well as details of my contributions. The first investigation explored the impact of LL on reproduction and organ allometries of giant TRrGH mice of various ages. These data were compared to normal mice, which served as a control. To determine fertility differences between female mice, litters were examined on a daily basis. In addition, organ masses were examined in both males and females to asses possible hormonal alterations in LL. Results indicate that LL exerts a negative impact on reproduction, which is more pronounced in TRrGH females than normals. This may have been a consequence of altered levels of reproductive hormones in that male and female TRrGH mice both exhibited increased gonad size in LL.

The purpose of the second paper was to determine if feeding behaviour was altered in LL-reared mice. In this report feeding rates were examined over a ten day period. In both LL and standard 12h dark:12h light photoperiod (LD), females ate more than males, and TRrGH mice ate less than their normal counterparts. The other transgenic strain examined, namely GLUT-4, which express double the amount of GLUT-4 glucose transporters, also ate less than normals. Of specific interest was the observed feeding reduction of LL-reared TRrGH females which would place a further energetic demand upon these animals.

The last article followed the behaviour of TRrGH males over a 24 hour period in LL. Behavioural time budgets (locomotion, exercise, feeding, drinking, sleep) and circadian feeding and sleep patterns were investigated and compared with data previously obtained in LD. Results suggest that TRrGH mice in LL are more behaviourally lethargic and sleep more. Circadian rhythms of feeding behavour and sleep are shifted by approximately 12 hours and peaks show reduced amplitude. These date indicate that TRrGH mice are not increasing energetic output in LL which would have removed energy from other physiological processes such as reproduction.

Finally, the *Concluding Discussion and Genral Summary* draws together the overall implications of the three studies and suggest areas which demand further research.

SECTION I

GENERAL INTRODUCTION

1

GENERAL INTRODUCTION

1.1 Transgenics and Methods of Gene Transfer

Genetic engineering of the genome is a powerful tool for probing genomic and neuroendocrinological regulation in higher eukaryotes (Gordon and Ruddle 1981). Only recently has this technology been developed. There are two main methods by which foreign DNA may be stably introduced into the germ line of mammals. One method involves the genetic manipulation of embryonic stem cells, or retroviral integration into early developing embryos. The latter is currently the procedure of choice, and is the only proven procedure for integrating foreign gene constructs into livestock. This involves the microinjection of the exogenous genes of interest into the pronucleus of fertilized oocytes (Babinet *et al.* 1989; Boyd and Samid 1993; Gordon 1993; Evans *et al.* 1994). This allows large gene constructs to be introduced into the genome. A disadvantage is that the identity of the chromosome within which the constructs are integrated remains unknown (Palmiter and Brinster 1986).

Our transgenic GH mice (TRrGHm) were produced by microinjection of fusion genes (metallothionein promoters coupled to rat growth hormone (rGH) structural genes) into the male pronuclei of fertilized mouse eggs, followed by implantation into pseudopregnant foster mothers. Figure 1.1 summarizes the basic steps utilized to establish transgenic lines of TRrGH mice. The result is that mice grow twice as fast as normal mice and achieve double normal body sizes (Palmiter *et al.* 1982).

1.2 The Principle of Allocation

The "principle of allocation" is fundamental to theories of life-history strategies and their evolution (Sibly and Calow 1986; Rollo 1994). It applies to the partitioning of

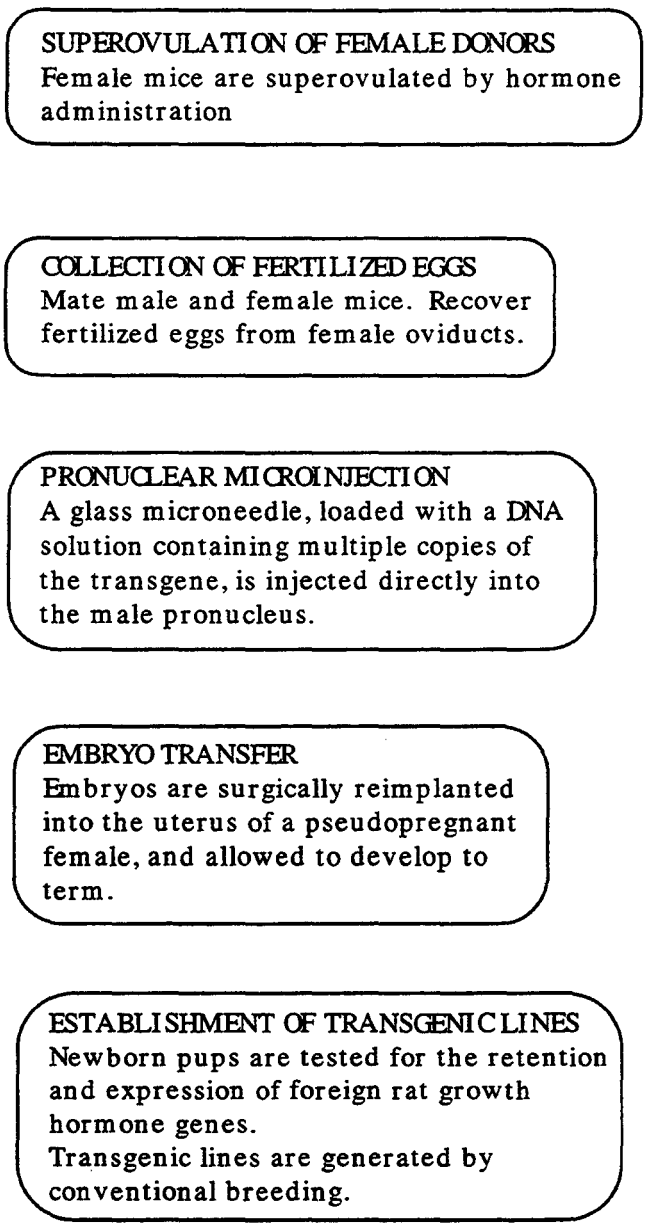

Figure 1.1. Production of transgenic GH mice by pronuclear microinjection.

available resources among competing physiological processes such as reproduction, behaviour, defense, growth, storage, and maintenance. Since resources are ultimately limited, the amplification of one process may have negative impacts (trade-offs) on other processes. In TRrGHm, the amount of resources put into growth is elevated. Therefore, if feeding is not increased, other processes must be traded-off to compensate. Reproduction, being highly dependent upon available resources, is suppressed in TRrGHm. Behaviour, longevity, and thermoregulation are also compromised (Kajiura and Rollo 1994; Lachmansingh and Rollo 1994; Rollo *et al.* 1997).

Several factors may conceal or offset trade-offs. Short-term investigations may fail to resolve trade-offs if organisms utilize storage reserves. In addition, organisms may vary in quality due to maternal influences, age, genetic variation, phenotypic plasticity, inbreeding, disease, or mutations, so some are better at everything and some are worse. Furthermore, it may be difficult to detect trade-offs if two components are enhanced, while a third feature is simultaneously compromised.

1.3 Basic Reproductive Endocrinology

The ovarian function across estrous cycles depends completely on the gonadotropic hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted by the anterior pituitary gland. Ovaries that are not stimulated by these hormones remain completely inactive. Pulses and cycles of FSH and LH (driven by hypothalamic luteinizing hormone releasing hormone (LHRH)) drive cyclic ovarian changes.

FSH regulates the early stages of follicular development in the ovary, after which accelerated growth of the follicle occurs. This accelerated growth involves a positive feedback loop. The follicle secretes estrogens, which in turn cause the number of FSH receptors on the follicle to increase. This causes follicular growth and further secretion of estrogens.

LH is necessary for final follicular growth and ovulation. Without this hormone, even though large quantities of FSH are available, the follicle will not progress to the stage of ovulation. Just before ovulation, LH levels rise six- to tenfold above normal basal levels. LH has a specific effect on the follicle causing it to secrete more progesterone and less estrogen. At this point ovulation occurs. After ovulation LH causes the cells surrounding the ovum to enlarge and become filled with lipid inclusions that give a yellowish appearance. This process is called*luteinization*, and the total mass of cells together is called the *corpus luteum.* In rodents, prolactin (PRL) is necessary to maintain the corpus luteum, which is responsible for producing large quantities of progesterone and estrogen. The most important function of progesterone is to induce secretory changes in the uterine endometrium, thus preparing the uterus for implantation of the fertilized ovum.

1.4 Reproduction and Transgenic Mice Models

Considerable recent research has addressed the effects of growth hormone (GH) on reproduction. Many strains of TRGHm models (rat GH, bovine GH, human GH, ovine GH) have been examined, all of which show reproductive impairment to varying degrees (Bartke *et al.* 1988; Naar *et al.* 1991; Pomp *et al.* 1995). For example, mice with a phosphoenolpyruvate decarboxykinase (PEPCK) promoter are more infertile than those with MT promoters, and human GH mice show greater reproductive impairment than rat GH mice. Reproductive deficiency generally involves luteal failure (Bartke *et al.* 1988; Naar *et al.* 1991; Cecim *et al.* 1995). This is significant since luteal function is required for the maintenance of early gestation as it is the principle source of progesterone and estrogen (Norman and Litwack 1987). Transgenic GH mice are hypoprolactinemic, with the exception of human GH mice which are considered hyperprolactinemic because human GH has PRL-like impacts in rodents (Bartke *et al.* 1994). In all cases, however, there is an absence of the mating-induced surges of prolactin (PRL) (Cecim *et al.* 1995). Since PRL

is an essential component of the luteotropic complex in rodents (Smith *et al.* 1975), its absence causes luteal failure. The absence of mating-induced PRL surges results from increased tuberoinfundibular dopaminergic (DA) activity (Steger *et al.* 1991; Cecim *et al.* 1995). Normally, DA activity decreases during PRL surges, but in transgenic GH mice this is not the case. Reproductive inhibition can be overcome by exogenous supplements of progesterone (Bartke *et al.* 1988), thereby mimicking the actions of the corpus luteum. In TRrGHm with only moderate reproductive impairment, high energy diets improve or restore fertility (Murray and Pomp 1995; Rollo *et al.* 1997).

1.5 Effects of Constant Light and Nutrition on Reproduction

Rearing rats and mice in constant light (LL) alters gonadotropin levels, organ masses, and estrous cycles (Fiske 1941; Daane and Parlow 1971; Mos 1976). For example LL-reared rats and mice show increased spleen and adrenal masses. Ovary mass initially increased in size but, after chronic exposure for a few months, ovaries became atrophic (Fiske 1941; Mos 1976). Gonadotropins, such as FSH and hormones, such as PRL also increase in LL treated rats and mice (Fiske 1941; Vaticon *et al.* 1980). LL treatments may cause females to enter persistent estrous with either delay or failure of ovulation (Fiske 1941; McCormack and Sridaran 1978; Campbell and Schwartz 1980). However, treatment with human chorionic gonadotropin and progesterone induced ovulation (Singh and Greenwald 1967).

Gonadotropin changes in LL have been applied to an advantage in rodents. Piacsek and Meites (1967) found decreased FSH, LH, and PRL release due to underfeeding in rats. These depressive effects of underfeeding were offset by LL. Rollo *et al.* (1997) and Murray and Pomp (1995) also demonstrated an interaction between dietary energy levels and reproductive performance in TRGHm. Recently our laboratory synthesized the literature and interpreted the reproductive failure as possibly reflecting a state of energetic

stress leading to the observed hormonal disregulation of fertility (Rollo *et al.* 1997). Based on this evidence we hypothesized that LL photoperiods might offset suppressed fertility in TRrGHm, a result that could have vast implications for production of transgenic livestock.

1.6 Stress and Reproduction

Chronic stress results in a release of adrenocorticotropic hormone (ACTH) from the pituitary gland, stimulated by corticotropic releasing hormone (CRF) release (corticosterone in rodents). ACTH then activates the adrenal cortex to release various steroids which regulate energy availability and allocation (deCatanzaro and MacNiven 1992; Rollo *et al.* 1997). In addition, other hormones such as gonadal steroids, or neurotransmitters such as serotonin may be altered (deCatanzaro and MacNiven 1992). During stress, corticosterone levels in mice are elevated (Barlow *et al.* 1974). Sillence and Etherton (1989) also demonstrated increased corticosterone and ACTH levels in rats injected with porcine GH. This correlated with results obtaines by Cecim *et al.* (1991) and Bartke *et al.* (1994) who observed increased corticosterone and ACTH levels in TRGHm. This would suggest that the stress axis (hypothalamic-pituitary-adrenal axis) is elevated in these mice. Hypersecretion of ACTH is strongly associated with reproductive disfunction (Kittinger *et al.* 1980). Estrogens may also be released from the adrenal and/or ovaries during stress effectively blocking reproduction (deCatanzaro and MacNiven 1992; deCatanzaro *et al.* 1994).

Adrenal mass is generally correlated with steroid and ACTH synthesis, and LL treated normal mice have increased mass-specific adrenal mass (Mos 1976). Transgenic GH mice also show increased absolute adrenal mass in standard 12h dark:12h light (LD) photoperiod (Cecim *et al.* 1991). Therefore it is feasible that LL treatment of normal mice, and transgenic GH mice hormonally simulates stress. The hypothalamic-pituitary-adrenal (HPA) axis and GH axis are counterregulatory, and the increased HPA axis function in

TRGHm is compensatory to chronic GH elevations. Regardless, these mice express a phenotype consistent with GH elevation. Consequently it is not possible to infer the consequences of LL on reproductive functions.

In conclusion, some evidence suggests that LL might alleviate reproductive suppression of energetically stressed mammals, but other evidence suggest it could aggravate problems of infertility. One goal of the current thesis was to assess these alterations.

SECTION II

IMPACT OF CONSTANT LIGHT ON FERTILITY AND ORGAN ALLOMETRY IN GIANT TRANSGENIC GROWTH HORMONE MICE: FURTHER SUPPRESSION OF FERTILITY AND ALTERED ORGAN ALLOMETRY

2

2.1 RATIONALE AND OBJECTIVES

This thesis describes the physiological resource allocation strategy of transgenic mice expressing mulitiple copies of rat GH genes. The main objective of this study was to examine the design of transgenic rat GH mice (TRrGHm) by comparing them to normal mice, and to use this data to assess possible causes of the reproductive impairment observed in these mice. Our laboratory investigated three areas of organismal function to determine how increased growth impacts various processes. We also examined the impact of continuous light on these processes. This required monitoring of i) life-history features (body size, fecundity, organ sizes), ii) feeding rates, and iii) behavioural time budgets (sleeping, locomotion, wheel running, feeding, drinking).

The present study focused on the first component of organism design, specifically on the impact of excess growth and continuous light on fertility and organ allometries. By placing mice in constant light, we hoped to improve fertility altering their hormonal balance. These ideas were based on studies demonstrating restored reproduction in underfed rats reared in constant light. Organs displaying altered mass-specific sizes can be an indication of altered hormone production and release. Hormonal alterations between light treatments were inferred from organ allometries. By evaluating the impact of photoperiod on fertility, we hoped to obyain a simple method for improving fertility of transgenic GH livestock, which exhibit similar physiological disfunctions as our TRrGHm.

2.2 CLARIFICATION OF CONTRIBUTIONS

This study was proposed by C. David Rollo, as a component of a larger research endeavor examining altered allocation and functions in TRrGHm. The current study examined the impact of constant light on reproduction, organ allometries, feeding behaviour, and activity of TRrGHm and normal controls of both sexes.

My contributions were as follows:

1. **Intellectual Contributions:**

(i) Review of scientific literature on transgenic GH animals, impacts of constant light on reproduction in rodents, and neuroendocrinological data was conducted largely by myself.

(ii) The theories and ideas presented in this thesis were synthesized jointly by Dr. C.D. Rollo and myself.

2. **Standard Animal Care of Main Breeding Colonies:**

All aspects of animal care were performed in conjunction with Dr. L. Kajiura.

Status of Health

(i) The status of health was checked for all animals daily. Cages were inspected for dead animals. Identification numbers, sex, birthdate, date of death, coat colour, strain (transgenic or normal), and diet treatment were noted for the deceased.
I was specifically responsible for culling the colony, and euthanizing any ill or injured mice.

(ii) Cages were checked daily for pregnant females and new litters. Identification numbers of parents, sex, coat colours, strain (transgenic or normal), birthdate of

offspring, and offspring body mass were recorded to complile data on the reproduction for transgenic and normal strains. At 20 days of age, mice were weaned to form new breeding groups or to be used for experimental purposes.

Food

Cage hoppers were stocked daily with standard rodent food pellets (LabDiet®, No. 5001, PMI Feed Inc., APPENDIX A) provided *ad libitum.*

Water

Water bottle were topped up daily. All bottles were washed with detergent and sterilized at three-day intervals.

Animal Breeding Cages

Cages (length x width x height = 28 x 17.5 x 5 cm), which contained 1 male and 4 females were washed, disinfected, and bedding material (wood chips) were replaced every 3 days.

3. Maintenance of Animal Quarters:

Floors, holding racks, and bench tops were washed and disinfected daily. Room temperature and photoperiod (L:D 12h:12h; L:L 24h) were also checked each day.

4. Experimental Design, Preparation, Data Documentation, and Statistical Analyses for Fertility and Organ Allometry Study:

(i) Monitoring of litters daily.

(ii) Sacrificing and weighing of mice for dissection.

(iii) Dissection of mice, drying and weighing of organs. Volunteers Grace and Chris were very helpful during these procedures.

(iv) Statistical comparisons (ANOVA, ANCOVA) were conducted using JUMP and MINITAB.

2.3

Impact of constant light on fertility and organ allometry in giant transgenic growth hormone mice: further suppression of fertility and altered organ allometry.

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2.4 ABSTRACT

Genetically engineered TRrGHm possess multiple copies of rat GH genes resulting in doubled growth rates. This excessive allocation of resources into growth results in reduced female fertility possibly due to a decrease in reproductive resources. To discover if photoperiod could improve fertility, mice were chronically exposed to 24h light (LL), and litters monitored daily. Individual dry organ masses were compared in sexes and between kinds of mice in LL and the standard 12h dark:12h light (LD) light regimen. Female TRrGH mice exhibited a 50% ($p<0.05$) decrease in fertility in LL. Normal females expressed a 14% decline ($p<0.05$). TRrGH males were 207% heavier than normal males in LD, and 182% heavier in LL. TRrGH females weighed 164% more than normal females in LD, and 142% more in LL. On a mass specific basis, TRrGH females showed a 131 percent increase in combined ovary mass in LL. A significant reduction in thymus and heart mass were also exhibited. No significant differences were observed in normal males or females between light treatments. TRrGH males showed increased testes size (113%) in LL. Both strains of mice exhibited a larger combined adrenal mass in females in both LL and LD, suggesting increased stress levels. Organ asymmetry assesses the difference in size between the right and left side of paired organs. Female TRrGHm exhibited greater kidney asymmetry in LL than normal females. No other asymmetry data for TRrGHm was significant. This lack of asymmetry in TRrGHm is suggestive that GH distortion does not cause developmental instability. The excessive reduction in fertility, and changes in mass specific organ masses infer hormonal alterations. These changes were accentuated in TRrGH mice.

2.5 INTRODUCTION

Genetic engineering of the genome of organisms is a useful tool providing insight into genomic and neuroendocrinological regulation in higher eukaryotes (Gordon and Ruddle 1981). Palmiter *et al.* (1982) produced the first transgenic GH mice by microinjecting eggs with metallothionein rat fusion genes (TRrGH). These mice showed extraordinarily high levels of the fusion mRNA in their liver, and a correlation was seen between the fusion mRNA and growth levels (Palmiter *et al.* 1982). GH levels in some TRrGH mice were as much as 800-fold higher than their unaffected littermates (Palmiter *et al.* 1982). Recently much work has been found on the effects of excess GH on reproduction. Many levels of transgenic mice models (eg. bovine GH, human GH, ovine GH) have been examined to determine the underlying mechanisms involved in the associated infertility. Reproductive deficiency results from luteal failure (Bartke *et al.* 1988; Naar *et al.* 1991; Cecim *et al.* 1995). A study done by Cecim *et al.* (1995) demonstrated that an absence in the mating-induced prolactin (PRL) surge is the cause of luteal failure. This occurs due to increased tuberoinfundibular dopaminergic activity (Steger *et al.* 1991; Cecim *et al.* 1995).

Evolutionary theory of reproductive tactics is based on three variables -- reproductive effort, costs of reproduction, and selection as a process of demographic optimization (Williams 1966). With respect to costs, limited resources must be allocated to certain areas (Tuomi *et al.* 1981) such as growth, for example. If all resources are put towards somatic growth, little is left to allocate to other areas. One consequence of this may be a decrease in reproduction (Tuomi *et al.* 1981). Therefore reproductive disfunction in TRrGH mice may partially be a result of ill-proportioned resource allocation. This would be exacerbated since Kajiura and Rollo (1994) discovered that TRrGH males fail to adjust food intake to compensate for increased growth. Processes which seem to be affected by elevated growth

include behaviour, longevity, thermogenesis, and reproduction (Lachmansingh and Rollo 1994).

It has been demonstrated that rearing rats and mice in LL alters gonadotropin levels, organ masses, and estrous cycling (Mos 1976; Fiske 1941). Gonadotropins, such as follicle-stimulating hormone (FSH), and hormones such as PRL increase in LL in rats and mice (Fiske 1941; Vaticon *et al.* 1980). Gonadotropin changes in LL have been applied to an advantage in rodents. Piacsek and Meites (1967) found decreased FSH, luteinizing hormone (LH), and PRL release due to underfeeding in rats. These depressive effects of underfeeding could be partially or completely overcome by exposure to LL. Based on the assumption that TRrGH mice are energetically limited, the purpose of this study was to determine if fertility could be improved by keeping TRrGH mice in LL. If so, then this would have profound implications for improving transgenic livestock which show similar reproduction impairment to the mice (Guthrie *et al.* 1991). Another consideration is that LL may put added stress on their system, thereby further reducing fertility. Organ allometry between TRrGH mice and normal controls in both light treatments were also examined to determine the effects of LL on TRrGH mice.

Darwin believed that deviations occur from the "law of symmetry", that is, anomalous asymmetries in normally symmetrical organisms (Palmer and Strobeck 1986). One kind of symmetry, fluctuating asymmetry, which refers to minute random deviations from perfect symmetry (VanValen 1962), has been advanced as a tool for inferring the health, quality, or developmental stability of organisms. Fluctuating asymmetry describes a specific pattern of bilateral variation - a frequency distribution of right minus left (R-L) differences whose mean is zero and whose shape does not depart from the normal. This study examined organ asymmetry in both photoperiods in an attempt to discern the developmental

stability of TRrGHm. It was hypothesized that TRrGHm would show developmental instability arising from the alteration of several physiological processes.

2.6 METHODS

2.6.1 *Animals Used*

TRrGHm (*Mus musculus,* transgenic strain Tg[MT-1,rGH], Bri2), were obtained from our breeding colony. This strain was engineered by Palmiter *et al.* (1982) through the microinjection of fusion genes (metallothionein-1 promoters fused to rat structural genes) into the pronuclei of fertilized mouse eggs. Several copies of rGH genes were incorporated into one chromosome, increasing plasma rGH levels 100-400 times above normal levels (Palmiter *et al.* 1982; Shea *et al.* 1987). Inheritence of these genes is Mendelian, so heterozygously transgenic fathers mated to normal females generate equal numbers of transgenic and normal animals. This provided control for genetic background.

Sixty heterozygously TRrGHm and 60 normal controls were dissected. Eight different groups were compared, four in 12h dark:12h light (LD) and four in 24h light (LL). The four groups in each light treatment consisted of 15 transgenic males, 15 normal males, 15 transgenic females, and 15 normal females.

2.6.2 *General Rearing Conditions*

Breeding groups (1male mated to 4 females) were housed in clear plastic cages (Length x width x height = 28 x 17.5 x 5 cm) at 22+/-2^0 C with a photoperiod of 12h dark:12h light or 24h light. A stainless steel hopper, placed over each container supported food pellets and a water bottle. All animals were fed *ad libitum* (Lab Diet®, No.5001, PMI Feed Inc.). Cages were bedded with BetaChips® (Hardwood Laboratory Bedding) and cleaned every 3 days. Each cage contained a bottle or plastic tube for behavioural enrichment. All protocols adhered to guidelines of the Canadian Council on Animal Care.

2.6.3 *Variables Investigated*

Litters were monitored daily in each photoperiod. The number of litters born to females in their first year of life was selected as the variable for comparison. Litter sizes were also monitored.

Dissections were performed on transgenic and normal males and females of varying ages from both light treatments. Mice were first sacrificed and then immediately weighed to obtain their wet mass. A ventromedial incision exposed the internal organs. Organs were then removed in the following order: spleen, gonads, adrenal glands, kidneys, thymus, heart. Organs were subsequently rinsed in distilled water and examined under a dissecting scope to remove any extraneous tissue, such as fat. Organs were placed on previously weighed foil sheets, labelled, and oven-dried at 60° C for 24 h. Organs were then weighed and the dry mass recorded. Uteri were not used due to variations in mass associated with the estrous cycle. Ten mice of varying ages were sacrificed and oven-dried to constant mass at 60° C to provide a wet mass to dry mass conversion factor. There was no difference in water content between the strains, but their was some variation with age. These conversion factors were than plotted against age to determine the appropriate conversion factor for each mouse (Appendix B).

The following variables were considered: organ dry mass (mg), body dry mass (g), sex, age (days) and light treatment.

2.6.4 *Analytical Methods*

Absolute organ masses were calculated in milligrams dry mass and relative organ masses in milligrams dry mass per gram dry body mass. Overall means (+/-S.D.) were calculated for each variable. Statistical analyses consisted of analysis of covariance (ANCOVA). Data from all eight experimental groups were analysed and compared.

2.7 RESULTS

Fertility for normal and TRrGH females in both photoperiods are provided in Figure 2.1. Organ dry masses for normal and TRrGH males and females in both photoperiods are provided in Tables 2.1 to 2.6. In addition, these data reveal the impacts of LL and transgenesis on organ allometry. Organ asymmetries for normal and TRrGH mice in both 12h dark:12h light (LD) and LL are provided in Tables 2.7 to 2.9.

2.7.1 *Fertility of Normal and TRrGH Females in LL vs LD*

There was a 14% reduction in fertility in LL for normal females (# females having at least one litter in their first year of life, LD: 47% versus LL: 40%, $p<0.05$), and a 50% reduction for TRrGH females (LD: 12.5% versus LL: 6.25%, $p<0.05$). TRrGH females had 69% fewer litters than normals in LD ($p<0.05$) and 68% in LL ($p<0.05$, Figure 2.1). No differences were observed in litter sizes between TRrGHm and normals.

2.7.2 *Normal versus TRrGH Mice in LD*

Male TRrGH mice were 207% heavier than normal males (13.988 +/- 1.709 versus 6.759 +/- 1.361 g, $p<0.000$), and female TRrGH mice were 164% heavier than normal females (12.939 +/- 1.728 versus 7.907 +/- 1.378 g, $p<0.000$). On a mass-specific basis, male TRrGH mice had a combined testes mass only 64% the mass of normals' testes (2.348 +/- 0.262 versus 3.688 +/- 0.607 mg/g dry body mass respectively, $p<0.000$). TRrGH females had a combined ovary mass only 59% that of normal females (0.300 +/- 0.103 versus 0.506 +/- 0.170 mg/g dry body mass, $p<0.001$). Normal females had a combined kidney mass larger than those of TRrGH females (11.639 +/- 1.377 versus 10.160 +/- 1.309 mg/g dry body mass, $p<0.009$ respectively, Table 2.1). No other mass-specific differences were observed.

2.7.3 *Normal versus TRrGH Mice in LL*

Male TRrGH mice were 182% larger than normal males (12.865 +/- 1.724 versus 7.072 +/- 1.432 g dry mass respectively, $p<0.000$). TRrGH females were 142% larger than normal females (12.797 +/- 2.151 versus 6.273 +/- 1.515 g dry mass respectively, $p<0.000$). The testes mass of TRrGH males was smaller (74%) than normals on a mass-specific basis (2.653 +/- 0.401 versus 3.575 +/- 0.472 mg/g dry body mass, $p<0.000$). TRrGH males also exhibited a combined relative adrenal mass only 63% that of normal males (0.126 +/- 0.050 versus 0.201 +/- 0.116 mg/g dry body mass, $p<0.02$). There were no significant mass-specific differences observed between TRrGH females and normal females for any organ systems (Table 2.2).

2.7.4 *Normal versus TRrGH Females in LD versus LL*

No significant differences in body masses were observed between TRrGH or between normal females between light treatments, although normal females in LL were slightly smaller. Mass-specifically, TRrGH females has a combined ovary mass 131% larger in LL than LD (0.394 +/- 0.136 versus 0.300 +/- 0.103 mg/g dry body mass respectively, $p<0.05$). They also exhibited reduced heart mass (90%) in LL (4.635 +/- 1.298 versus 5.159 +/- 1.026 mg/g dry body mass respectively, $p<0.05$) as well as a reduced thymus (78%) size (3.261 +/- 0.929 versus 4.175 +/- 1.611 mg/g dry body mass respectively, $p<0.05$). No significant differences in relative organ sizes were observed in normal females between LL and LD (Table 2.3).

2.7.5 *Normal versus TRrGH Males in LD versus LL*

Body masses were not significantly different between LD and LL for either TRrGH and normal males, although LL-reared males appeared slightly smaller. TRrGH males had significantly larger testes (113%) in LL than in LD on a mass-specific basis (2.653 +/-

0.401 versus 2.348 +/- 0.262 mg/g dry body mass, $p<0.02$, Table 2.4). No other differences were observed.

2.7.6 *Normal versus TRrGH Males versus Females in LD*

Male TRrGH mice were significantly larger than TRrGH females (13.988 +/- 1.709 versus 12.939 +/- 1.728 g dry mass respectively, $p<0.000$). No significant differences were observed between normal males and normal females. Mass specifically, TRrGH females had a 223% larger combined adrenal mass that the TRrGH males (0.272 +/- 0.109 versus 0.122 +/- 0.048 mg/ g dry body mass respectively, $p<0.000$). Normal females demonstrated a combined adrenal mass 163% that of normal males (0.271 +/- 0.104 versus 0.166 +/- 0.045 mg/g dry body mass respectively, $p<0.000$). Normal females also exhibited significantly larger hearts than normal males (5.537 +/- 0.856 versus 5.248 +/- 0.595 mg/g dry body mass, $p<0.04$, Table 2.5).

2.7.7 *Normal versus TRrGH Males versus Females in LL*

The consistent difference in body mass between TRrGH males and females found in LD disappeared in LL (12.865 +/- 1.724 versus 12.797 +/- 2.151 g dry body mass respectively), however normal males were 113% larger than normal females in LL (7.072 +/- 1.432 versus 6.273 +/- 1.515 g dry body mass respectively, $p<0.001$). TRrGH males showed a reduced combined adrenal mass, 38% of the TRrGH females mass specifically (0.126 +/- 0.050 versus 0.330 +/- 0.101 mg/g dry body mass, $p<0.000$). No differences in adrenal mass were observed between normal males and females. The kidneys of TRrGH males were 122% larger than the females (12.327 +/- 2.419 versus 10.074 +/- 1.611 mg/g dry body mass respectively, $p<0.006$), and the thymus was also larger in the TRrGH males by 136% (4.428 +/- 0.718 versus 3.261 +/- 0.929 mg/g dry body mass respectively, $p<0.001$). Normal males had a combined kidney mass 115% greater than normal females in LL (12.722 +/- 1.356 versus 11.095 +/- 1.567 mg/g dry body mass respectively, $p<0.01$, Table 2.6).

2.7.8 *Organ Asymmetry*

Organ asymmetry is the difference in size between the right and left side of paired organs. Female TRrGH mice showed greater absolute kidney asymmetry than normal females in LL (6.057 +/- 4.938 versus 4.938 +/- 1.828 mg, $p<0.02$) but not in LD. Normal females exhibited a larger absolute adrenal asymmetry than normal males in LD (0.303 +/- 0.264 versus 0.076 +/- 0.078 mg, $p<0.001$) but nor in LL. Normal females demonstrated reduced kidney asymmetry in LL compared to LD (1.828 +/- 1.505 versus 3.692 +/- 2.600 mg, $p<0.002$. Tables 2.7 to 2.9).

2.8 DISCUSSION

Results suggest that infertility in female TRrGH mice may be exacerbated in LL. Pregnancy rates in the first year of life were reduced by 50% in LL (Figure 2.1, $p<0.05$). Normal females also demonstrated a significant reduction in prenancy rates in LL (Figure 2.1, $p<0.05$). Differences in organ weights were also detected in both transgenic and normal males and females between photoperiods. Such alterations in organ mass are indicators of altered hormone release. Altered levels of stress hormones, estrogens, and prolactin may have contributed to the increased reproductive deficiency in LL treated TRrGH and normal females.

Results indicate that TRrGH females were infertile compared to their normal siblings. Other kinds of TRGHm are also highly infertile (Bartke *et al.* 1994; Cecim *et al.* 1995; Murray and Pomp 1995). Smith *et al.* (1975) showed that PRL, along with FSH, forms the essential luteotropic complex required to maintain the corpus luteum (Day *et al.* 1980). Functional corpora lutea are responsible for the maintenance of early gestation and are the primary source of progesterone during this period. Without progesterone, implantation does not take place. This appears to be the case with transgenic GH mice in that reproductive inhibition can be overcome with exogenous progesterone supplements (Bartke *et al.* 1988). The hormone imbalance may be a direct result of reduced energy supplies. TRrGH males have been shown to eat less mass-specifically that normal males (Kajiura and Rollo 1994). Hence, their excessive allocation of energetic resources into growth, leaves less resources for other processes such as reproduction.

Kittinger *et al.* (1980) found that increased ACTH induces hyperprolactinemia and transgenic GH mice show increased ACTH levels (Bartke *et al.* 1994). Since TRrGHm are hypoprolactinemic, ACTH either: 1) may not be exerting an effect on PRL levels, or 2) the effect of ACTH in negligible compared with other mechanisms exerting an effect on PRL

levels. The latter seems more probable, and increased tuberoinfundibular dopaminergic activity appears to be the other mechanism. Usually dopamine (DA) levels decline during PRL surges but in transgenic GH mice, DA levels remain elevated (Steger *et al.* 1991; Cecim *et al.* 1991).

When chronically exposed to LL, fertility is reduced. The TRrGH females exhibited a 50% reduction in fertility (Figure 2.1) when exposed to LL, whereas normal females demonstrated a 14% loss in fertility. The effect of LL on the estrous cycle of rats has been investigated in many reports (Fiske 1941; Singh and Greenwald 1976; McCormack and Sridaran, 1978; Campbell and Schwartz 1980). Short-term exposure improves fertility (Fiske 1941). Ovaries enlarge and gonadotropin levels increase. Long term exposure to LL leads to a fade-out of estrous cycles. Exposure to LL eventually induces persistent estrous, which is characterized by prolonged vaginal cornification and cystic follicular ovaries devoid of corpora lutea (Fiske 1941; Singh and Greenwald 1976). Lawton and Shwartz (1967) demonstrated decreased ovulation in LL treated rats. Estrogen levels are increased in LL (Fiske 1941; Piacsek and Meites 1967) and there is an absence of the preovulatory LH surge (Lawton and Schwartz 1967; McCormack and Sridaran 1978). Progesterone has been shown to synchronize the timing of LH discharge in female rats (Mann *et al.* 1976), Therefore alterations in progesterone levels may also have been present in LL. PRL levels have also been observed to increase in LL (Vaticon *et al.* 1980). This elevation could be in response to elevations in either serotonin, estrogen or ACTH. LL exposure may be accompanied by an increase in serotonin levels (Vaticon *et al.* 1980). Hoffmann and Cullin (1975) exhibited reduced pineal size (melatonin) in LL reared rats, but they showed that the pineal gland is not involved in induction of persistent estrous, perhaps since melatonin is only synthesized in the dark. Estrogen levels exert a positive influence on PRL levels (Vaticon *et al.* 1980; Chandrashekar *et al.* 1992), and estrogen levels are elevated in LL. ACTH also increases in LL which could induce

hyperprolactinemia in mice (Kittinger *et al.* 1980; Fischman *et al.* 1988). ACTH inhibits estrous cycling in mammals. Impacts may involve peripheral impacts on the ovary (deCatanzaro and MacNiven 1992), or central impacts regulating estrous cycles and energy allocation (Rollo *et al.* 1997). Adrenal hormones, such as corticosterone, decrease in LL (Fischman *et al.* 1988), which is unexpected given the normal trend of increased corticosterone being associated with increased estrogen (Kitay *et al.* 1971; Burgess and Handa 1992).

A number of mechanisms could have resulted in the observed fertility decrease of TRrGH females in LL. First, ACTH levels may have been further elevated by exposure to LL. Since TRrGH mice already exhibit increased ACTH levels (Bartke *et al.* 1994), a further increase could have been reproductively detrimental. TRrGH females may have shown decreased ovulation as well as implantation. Regardless, corticosterone, does not affect fertility in mammals (deCatanzaro and MacNiven 1992).

Stress and reproduction are mutually antagonistic (Grey 1971, Rollo *et al.* 1997). If LL induces a hormonal profile of stress, then reproductive deficits may increase (Rollo *et al.* 1997). For example, Fiske (1941) indicated that estrogen levels increase in LL treated rats, but after a time ovaries regress in size. No regression of the ovaries occured in TRrGH females with age (Figure 2.2) suggesting that estrogen levels may have remained elevated in LL. The adrenal gland is another source of estrogen, and during stress estradiol levels increase (Campbell *et al.* 1977; deCatanzaro and MacNiven 1992). Excess estrogens block pregnancy (e.g. birth control pills). Greenwald (1965) showed that estrogen induces luteal degeneration in the hamster. DeCatanzaro *et al.* (1992) demonstrated that estrogen antibodies reduce vulnerability to stress-induced failure of intrauterine implantation. Estrogenic substances have been found to have adverse effects on the migration of fertilized ova through fallopian tubes, with small doses leading to retention and subsequent degeneration of ova (deCatanzaro and MacNiven 1992). Central impacts on the LHRH

pulse generator are also implicated (Rollo *et al.* 1997). Other adrenal steroids may also have impacted on female fertility. DeCatanzaro and MacNiven (1992) indicated that androstenedione and dehydroepiandosterone are released during stress, and these can cause expulsion of fertilized eggs from the reproductive tract.

The mechanism underlying transgenic infertility is ultimately the absence of mating-induced PRL surges. LL does not appear to alleviate this. Basal PRL levels may have increased, as is seen in rats, however if the surges are still absent, fertility will not improve. In female rats, Mann *et al.* (1976) demonstrated increased PRL levels in LL, but peak concentrations of PRL attained were less than in LD. This suggests that, in LL, PRL rhythms are blunted. Altered serotonin rhythms are implicated.

Transgenic rat, bovine, or human GH mice all express altered organ masses (Shea *et al.* 1987; Cecim *et al.* 1991). Alterations of organ mass have also been demonstrated in rats and mice reared in LL (Fiske and Lambert 1962; Lawton and Schwartz 1967; Mos 1976). Both impacts were exhibited in this investigation, attesting to alterations in neuroendocrine balance.

Normal females in LL showed a non-significant trend to reduced body mass. A similar impact was obtained in rats (Hoffmann and Cullin 1975). Both male and female TRrGHm also demonstrated slight, but non-significant reductions in body mass in LL, although the effects were more pronounced in the males. A larger sample size may have resolved these trends. Estrogen is known to be altered in LL (Fiske 1941; Campbell and Schwartz 1980) and estrogen may impact strongly on growth. Indeed TRrGH females are consistently smaller than males and estrogens reduce body weight in female rats (Wade and Gray 1978; Ramirez 1981; Palmer and Gray 1986; Butera and Beikirch 1988). In male rats, testosterone inhibits food intake and decreases body weight (Wade and Gray 1978). TRrGH males exhibited increased relative testes mass in LL which may have been indicative of increased testosterone production.

When mass specific adrenal mass was examined, no differences were observed between females and light treatments. This agrees with results obtained by Shea *et al.* (1987) for animals in an LD photoperiod. This would suggest either: 1) hormones affecting adrenal output are not altered between treatments, 2) adrenal sensitivity to hormonal changes is reduced, or 3) an increase in production of some adrenal steroids counteracts decreased production of others. Both of the latter are possible. In rats treated with porcine GH, increased levels of adrenocorticotropic hormone (ACTH) are observed, but adrenal responsiveness to ACTH is reduced (Sillence and Etherton 1989). Therefore, even though ACTH and CORT levels are elevated in transgenic GH mice (Bartke *et al.* 1994) and in LL treated rats (Fischman *et al.* 1988), the adrenal may be desensitized in TRrGH females. It has also been demonstrated that LL impacts various adrenal hormones differently. For example, corticosterone levels decrease in LL (Fischman *et al.* 1988). In contrast, if continuous light acted as a stress, then increased levels of adrenal estrogens (Campbell *et al.* 1977; deCatanzaro and MacNiven 1992), androstenedione, or dehydroepiandosterone may have been produced (deCatanzaro and MacNiven 1992). Both TRrGH and normal males exhibited reduced adrenal mass compared to females. Females express higher circulating corticosterone and stress-induced corticosterone responses (Kitay 1961; Critchlow *et al.* 1963). Estrogens directly affect peptide and mRNA levels of corticotropin releasing hormone (CRF) (Haas and George 1988; Swanson and Simmons 1989; Bohler *et al.* 1990). Estrogens also facilitate ACTH release from the anterior pituitary (Coyne and Kitay 1969). Both CRF indirectly, and ACTH directly, affects adrenal hormone release.

In LD, TRrGH females exhibited smaller ovary masses than normals on a mass-specific basis (Table 2.1). One reason may have been the reduced FSH or progesterone observed in TRGHm (Tang *et al.* 1993; Bartke *et al.* 1994; Cecim *et al.* 1995). Unlike normals, TRrGH females in LL expressed larger mass-specific ovaries compared with LD

(Table 2.3). Elevated FSH, which results in estrogen release, increases in LL treated rats (Fiske 1941; Campbell and Schwartz 1980). Therefore, the larger ovary size observed in LL treated TRrGH females versus LD treated TRrGH females may have resulted from elevated estrogen. Ovary size in the normals was greater in LD than LL but this was not statistically resolved. This agreed with Hoffmann and Cullin (1975) who examined rats, but contradicts those of Mos (1973, 1976) who showed increased ovary size in LL treated mice. This could be explained since Mos used only young age-matched mice. Fiske (1941) showed that animals kept in LL expressed age-specific decreases in FSH and ovary mass. TRrGH mice may not exhibit this pattern since elevated FSH levels may be more stable with age due to chronically high GH production (Figure 2.2).

TRrGH mice exhibit increased relative kidney masses compared to normals (Shea *et al.* 1987; Wanke *et al.* 1992). Bovine GH mice have exhibited glomerular enlargement and progressive glomerularsclerosis. Eventually glomerular disfunction ensues which is evidenced by numerous intratubular protein casts (Brem *et al.* 1989; Wanke *et al.* 1991). Reduced longevity observed in transgenic GH mice may often result from renal failure (Brem *et al.* 1988; Quaife *et al.* 1989; Brem *et al.* 1989) . A reduction in relative thymus and heart sizes were observed in TRrGH females in LL. This suggests both decreased immune response as well as activity levels although GH is well known to increase immune response. Reduced heart size may have resulted from decreased behavioural activity (see Section IV).

Studies of growth rates demonstrate that fast growth (Danzmann *et al.* 1986; Arnold 1988; McKenzie and O'Farrell 1993; Moller *et al.* 1995; Bjorklund 1996; Moller 1997) and high fecundity (Chenuil 1991; Moller *et al.* 1996; Swaddle 1996; Agnew and Koella 1997) generally are associated with symmetrical phenotypes in various animals (i.e. high fitness). Our results suggest, however, that overall fitness is not associated with symmetry. TRrGHm show high levels of organ symmetry in both sexes and photoperiods (Tables 2.7

to 2.9), but decreased fertility and longevity (Kajiura and Rollo 1994). The symmetry observed in TRrGHm would suggest that these mice are developmentally stable, thereby undergoing stable development of their phenotype under a range of environmental conditions (Moller 1997). This contradicts the tenant of asymmetry theory (that symmetry can be used as a reliable biomarker of fitness), since LL-reared TRrGHm clearly demonstrated large reproductive deficiencies resulting from an altered environment. Therefore, fluctuating asymmetry does not reflect fitness levels in TRrGHm.

In conclusion, the present results demonstrate that infertility in female TRrGH and normal mice is aggravated by LL. The most likely mechanisms (that are not mutually exclusive) are: 1) absence of mating induced PRL surges, 2) increased adrenal (estrogen, CORT) hormones, and 3) a decreased ovulation resulting from disrupted estrous cycling. Measurements of reproductive and adrenal steroids, as well as gonadotropins, still have yet to be investigated. The present results attest that neuroendocrine balance is likely modified by photoperiod, with greater impacts on transgenics than normals. The effects of darkness, or short bursts of LL, on fertility could provide additional insight into the effects of photoperiod on reproduction, and perhaps practical applications to domestic livestock.

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FIGURE 2.1. Fertility of transgenic and normal females in their first year of life in both photoperiods. LL-reared transgenic females exhibited a 50% reduction in fertility ($p<0.05$). Normal females exhibited a 14% fertility reduction in LL ($p<0.05$). Transgenic females exhibited a 69% ($p<0.05$) and 68% ($p<0.05$) reduction in fertility when compared to normals in LD and LL repectively.

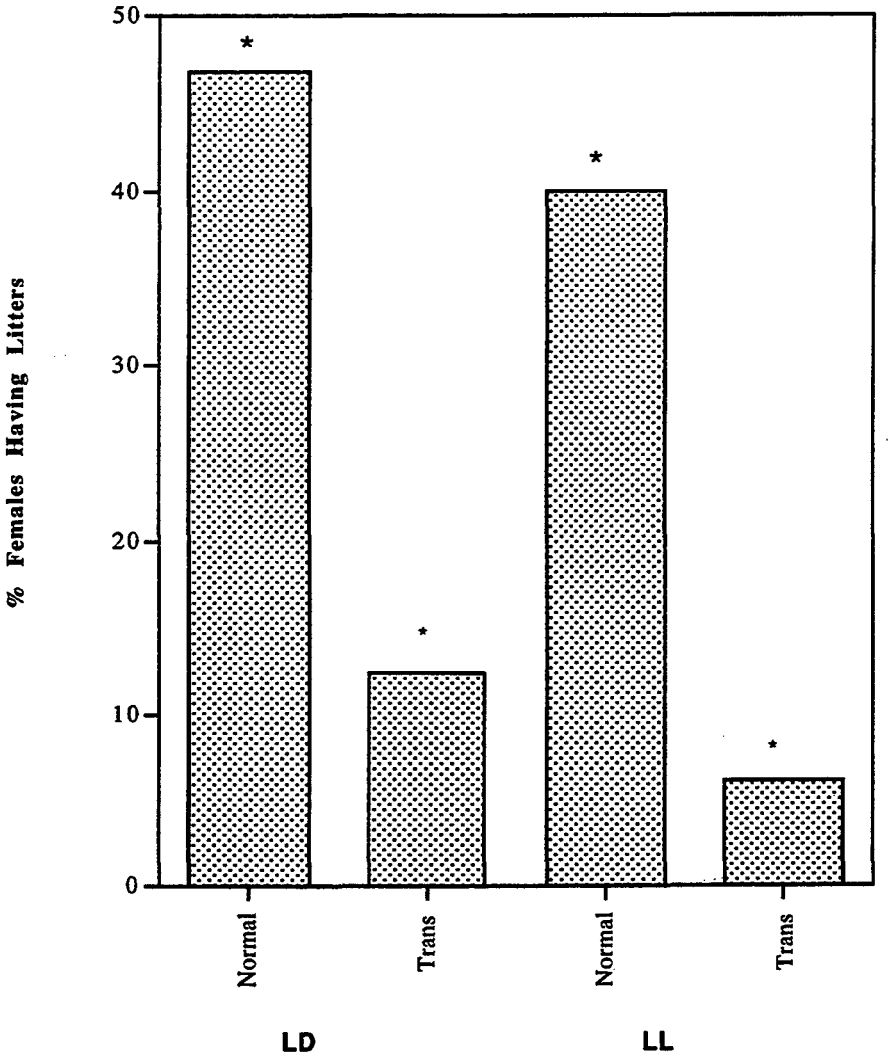
% Females Having Litters
50
40
30
20
10
0
*
*
*
*
Normal
Trans
Normal
Trans
LD
LL

FIGURE 2.2. Ovary size as a function of age in transgenic females in LL. No significant trends are seen.

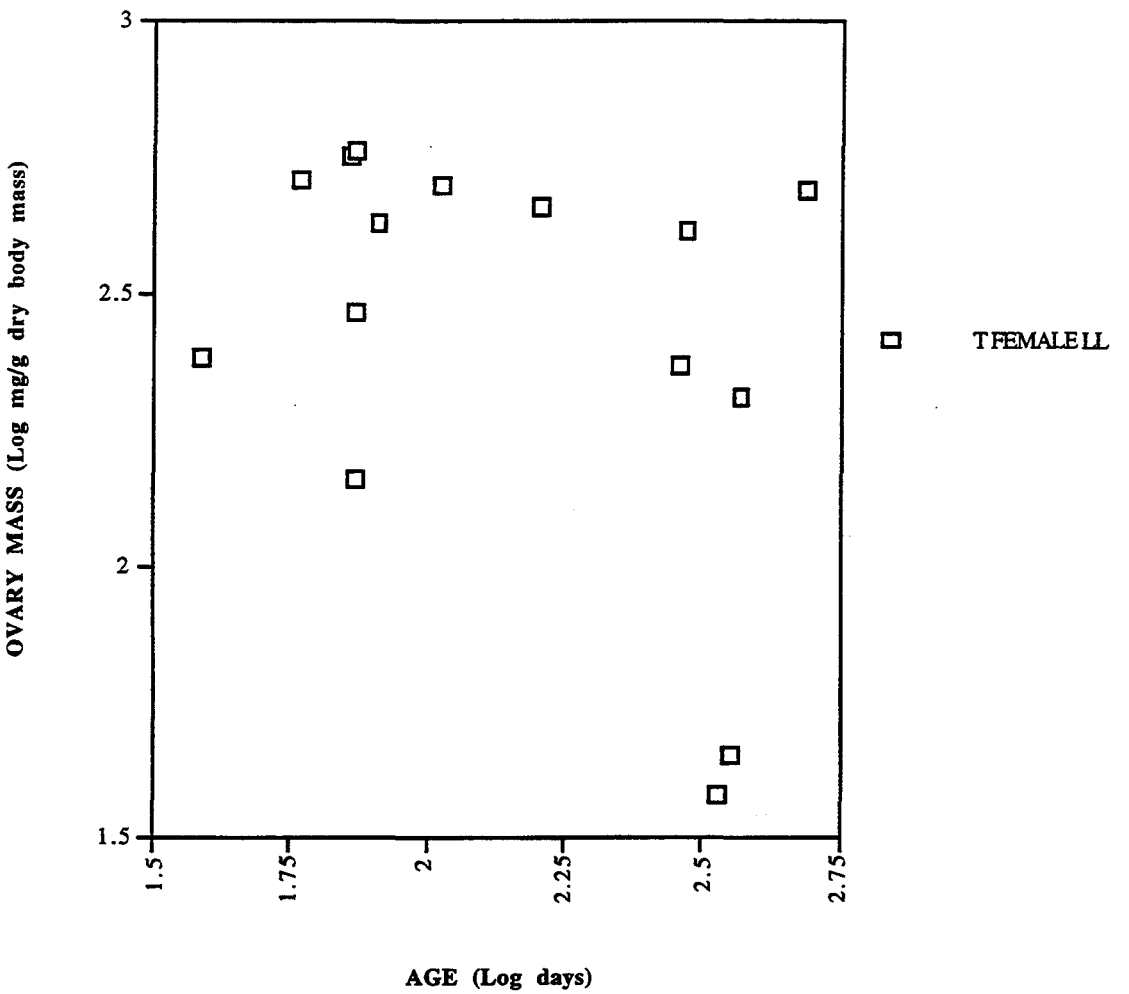
3
2.5
2
1.5
OVARY MASS (Log mg/g dry body mass)
1.5
1.75
2
2.25
2.5
2.75
AGE (Log days)
TFEMALELL

TABLE 2.1. Comparison of organ masses of transgenic versus normal A) males and B) females in 12h dark:12h light photoperiod. (*) indicates significance ($p<0.05$). Body mass is expressed in dry g, all other tissues in dry mg (absolute) or dry mg/g dry body mass (relative).

	A) MALES					B) FEMALES				
	Transgenic		Normal		%T/N	Transgenic		Normal		%T/N
	Absolute	S.D.	Absolute	S.D.		Absolute	S.D.	Absolute	S.D.	
Body(g)	13.988*	1.709	6.759*	1.361	206.9	12.939*	1.728	7.907*	1.378	163.6
Gonads	32.557*	3.091	24.714*	5.086	131.7	3.845	1.222	3.879	1.098	99.1
Adrenals	1.657*	0.546	1.078*	0.206	153.7	3.477*	1.488	2.057*	0.610	169.0
Kidneys	167.06*	29.73	88.50*	17.60	188.8	130.45*	17.79	90.89*	17.68	143.5
Spleen	51.665*	28.738	19.326*	3.491	267.3	43.385*	13.721	23.127*	6.879	187.6
Thymus	65.385*	12.820	31.884*	10.627	205.1	53.279*	15.677	32.474*	9.557	164.1
Heart	71.117*	13.140	35.111*	5.907	202.5	66.375*	14.711	43.302*	7.540	153.3
	Relative	S.D.	Relative	S.D.		Relative	S.D.	Relative	S.D.	
Gonads	2.348*	0.262	3.688*	0.607	63.7	0.300*	0.103	0.506*	0.170	59.3
Adrenals	0.122	0.048	0.166	0.045	73.5	0.272	0.109	0.271	0.104	100.0
Kidneys	11.933	1.543	13.143	1.332	90.8	10.160*	1.309	11.639*	1.377	87.3
Spleen	3.699	1.900	2.903	0.471	127.4	3.345	0.854	2.961	0.788	113.0
Thymus	4.691	0.821	4.753	1.578	98.7	4.175	1.611	4.159	1.205	100.0
Heart	5.135	1.080	5.248	0.595	97.8	5.159	1.026	5.537	0.856	93.2

TABLE 2.2. Comparison of organ masses of transgenic versus normal A) males and B) females in 24h light photoperiod. (*) indicates significance ($p<0.05$). Body mass is expressed in dry g, all other tissues in dry mg (absolute) or dry mg/g dry body mass (relative).

	A) MALES (LL)					B) FEMALES (LL)				
	Transgenic		Normal		%T/N	Transgenic		Normal		%T/N
	Absolute	S.D.	Absolute	S.D.		Absolute	S.D.	Absolute	S.D.	
Body(g)	12.865*	1.724	7.072*	1.432	181.9	12.797*	2.151	6.273*	1.515	142.0
Gonads	34.499*	4.627	25.091*	5.305	137.5	5.112*	2.026	2.808*	1.505	182.1
Adrenals	1.636	0.728	1.451	1.042	112.7	4.250*	1.603	1.745*	0.762	243.6
Kidneys	156.02*	21.63	89.41*	17.32	174.5	127.36*	22.03	68.56*	15.19	185.8
Spleen	47.563*	22.562	18.728*	8.906	254.0	47.822*	14.353	20.615*	7.078	232.0
Thymus	47.563*	11.468	32.098*	10.587	176.9	41.657*	13.364	23.661*	6.257	176.1
Heart	60.197*	14.713	36.345*	6.689	165.6	68.685*	13.883	34.631*	9.341	198.3
	Relative	S.D.	Relative	S.D.		Relative	S.D.	Relative	S.D.	
Gonads	2.653*	0.401	3.575*	0.472	74.2	0.394	0.136	0.434	0.165	90.8
Adrenals	0.126	0.050	0.201	0.116	62.7	0.330	0.101	0.284	0.112	116.2
Kidneys	12.327	2.419	12.722	1.356	96.9	10.074	1.611	11.095	1.567	90.8
Spleen	3.785	1.985	2.705	1.224	140.0	3.759	1.011	3.298	0.796	114.0
Thymus	4.428	0.718	4.523	0.971	97.9	3.261	0.929	3.900	1.123	83.6
Heart	4.913	1.151	5.243	0.993	93.7	4.635	1.298	5.655	1.445	82.0

TABLE 2.3. Comparison of organ masses in 12hr dark:12hr light versus 24h light photoperiod in A) transgenic and B) normal females. (*) indicates significance ($p<0.05$). Body mass is expressed in dry g, all other tissues in dry mg (absolute) or dry mg/g dry body mass (relative).

	A) TRANSGENIC					B) NORMAL				
	LD		LL		%LD/LL	LD		LL		%LD/LL
	Absolute	S.D.	Absolute	S.D.		Absolute	S.D.	Absolute	S.D.	
Body(g)	12.939	1.728	12.797	2.151	101.1	7.907	1.378	6.273	1.515	126.0
Gonads	3.845*	1.222	5.112*	2.026	75.2	3.879	1.098	2.808	1.505	138.1
Adrenals	3.477	1.488	4.250	1.603	81.8	2.057	0.610	1.745	0.762	117.9
Kidneys	130.45	17.79	127.36	22.03	73.3	90.89*	17.68	68.56*	15.19	132.6
Spleen	43.385	13.721	47.822	14.353	90.7	23.127	6.879	20.615	7.078	112.3
Thymus	53.279*	15.677	41.657*	13.364	127.9	32.474	9.557	23.661	6.257	137.2
Heart	66.375	14.711	68.685	13.883	96.7	43.302	7.540	34.631	9.341	125.0
	Relative	S.D.	Relative	S.D.		Relative	S.D.	Relative	S.D.	
Gonads	0.300*	0.103	0.394*	0.136	76.1	0.506	0.170	0.434	0.165	116.6
Adrenals	0.272	0.109	0.330	0.101	82.4	0.271	0.104	0.284	0.112	95.4
Kidneys	10.160	1.309	10.074	1.611	100.8	11.639	1.377	11.095	1.567	104.9
Spleen	3.345	0.854	3.759	1.011	89.0	2.961	0.788	3.298	0.796	89.8
Thymus	4.175*	1.611	3.261*	0.929	128.0	4.159	1.205	3.900	1.123	106.6
Heart	5.159*	1.026	4.635*	1.298	111.3	5.537	0.856	5.655	1.445	97.9

TABLE 2.4. Comparison of organ masses in 12hr dark:12hr light vs. 24hr light photoperiod in A) transgenic and B) normal males. (*) indicates significance ($p<0.05$). Body mass is expressed in dry g, all other tissues in dry mg (absolute) or dry mg/g dry body mass (relative).

	A) TRANSGENIC					B) NORMAL				
	LD		LL		%LD/LL	LD		LL		%LD/LL
	Absolute	S.D.	Absolute	S.D.		Absolute	S.D.	Absolute	S.D.	
Body(g)	13.988	1.709	12.865	1.724	108.7	6.759	1.361	7.072	1.432	95.6
Gonads	32.557	3.091	34.499	4.627	94.4	24.714	5.086	25.091	5.305	98.5
Adrenals	1.657	0.546	1.636	0.728	101.3	1.078	0.206	1.451	1.042	74.3
Kidneys	167.06	29.73	156.02	21.63	107.1	88.50	17.60	89.41	17.32	99.0
Spleen	51.665	28.738	47.563	22.562	108.6	19.326	3.491	18.728	8.906	103.2
Thymus	65.385	12.820	56.783	11.468	115.1	31.884	10.627	32.098	10.587	99.3
Heart	71.117*	13.140	60.197*	14.713	118.1	35.111	5.907	36.345	6.689	96.6
	Relative	S.D.	Relative	S.D.		Relative	S.D.	Relative	S.D.	
Gonads	2.348*	0.262	2.653*	0.401	88.5	3.688	0.607	3.575	0.472	103.2
Adrenals	0.122	0.048	0.126	0.050	96.8	0.166	0.045	0.201	0.116	82.6
Kidneys	11.933	1.543	12.327	2.419	96.8	13.143	1.332	12.722	1.356	103.3
Spleen	3.699	1.900	3.785	1.985	97.7	2.903	0.471	2.705	1.224	107.3
Thymus	4.691	0.821	4.428	0.718	105.9	4.753	1.578	4.523	0.971	105.2
Heart	5.135	1.080	4.913	1.151	104.5	5.248	0.595	5.243	0.993	100.1

TABLE 2.5. Comparison of organ masses of A) transgenic and B) normal males and females in 12h dark:12h light photoperiod. (*) indicates significance ($p<0.05$). Body mass is expressed in dry g, all other tissues in dry mg (absolute) or dry mg/g dry body mass (relative).

	A) TRANSGENIC					B) NORMAL				
	Males		Females		%M/F	Males		Females		%M/F
	Absolute	S.D.	Absolute	S.D.		Absolute	S.D.	Absolute	S.D.	
Body(g)	13.988*	1.709	12.939*	1.728	108.1	6.759	1.361	7.907	1.378	85.5
Adrenals	1.657*	0.546	3.477*	1.488	47.7	1.078*	0.206	2.057*	0.610	52.4
Kidneys	167.06*	29.73	130.45*	17.79	128.1	88.50*	17.60	90.89*	17.68	97.4
Spleen	51.665	28.738	43.385	13.721	119.1	19.326	3.491	23.127	6.879	83.6
Thymus	65.385*	12.820	53.279*	15.677	119.0	31.884	10.627	32.474	9.557	98.2
Heart	71.117	13.140	66.375	14.711	107.1	35.111	5.907	43.302	7.540	81.2
	Relative	S.D.	Relative	S.D.		Relative	S.D.	Relative	S.D.	
Adrenals	0.122*	0.048	0.272*	0.109	44.9	0.166*	0.045	0.271*	0.104	61.3
Kidneys	11.933*	1.543	10.160*	1.309	117.5	13.143	1.332	11.639	1.377	112.9
Spleen	3.699	1.900	3.345	0.854	110.6	2.903	0.471	2.961	0.788	98.0
Thymus	4.691	0.821	4.175	1.611	112.4	4.753	1.578	4.159	1.205	114.3
Heart	5.135	1.080	5.159	1.026	99.5	5.248*	0.595	5.537*	0.856	94.8

TABLE 2.6. Comparison of organ masses of A) transgenic and B) normal males and females in 24hr light photoperiod. (*) indicates significance ($p<0.05$). Body mass is expressed in dry g, all other tissues in dry mg (absolute) or dry mg/g dry body mass (relative).

	A) TRANSGENIC (LL)					B) NORMAL (LL)				
	Males		Females		%M/F	Males		Females		%M/F
	Absolute	S.D.	Absolute	S.D.		Absolute	S.D.	Absolute	S.D.	
Body(g)	12.865	1.724	12.797	2.151	100.5	7.072*	1.432	6.273*	1.515	112.7
Adrenals	1.636*	0.728	4.250*	1.603	38.5	1.451	1.042	1.745	0.762	83.2
Kidneys	156.02*	21.63	127.36*	22.03	122.5	89.41*	17.32	68.56*	15.19	130.4
Spleen	47.563	22.562	47.822	14.353	99.5	18.728	8.906	20.615	7.078	90.8
Thymus	56.783*	11.468	41.657*	13.364	136.3	32.098*	10.587	23.661*	6.257	135.7
Heart	60.197	14.713	68.685	13.883	87.6	36.345	6.689	34.631	9.341	104.9
	Relative	S.D.	Relative	S.D.		Relative	S.D.	Relative	S.D.	
Adrenals	0.126*	0.050	0.330*	0.101	38.2	0.201	0.116	0.284	0.112	70.5
Kidneys	12.327*	2.419	10.074*	1.611	122.4	12.722*	1.356	11.095*	1.567	114.7
Spleen	3.785	1.985	3.759	1.011	100.7	2.705	1.224	3.298	0.796	82.0
Thymus	4.428*	0.718	3.261*	0.929	135.8	4.523	0.971	3.900	1.123	116.0
Heart	4.913	1.151	4.635	1.298	106.0	5.243	0.993	5.655	1.445	92.7

TABLE 2.7. Comparison of absolute right and left organ mass difference (mg) in transgenic versus normal A) males and B) females in 12h dark:12h light and 24h light photoperiods. (*) indicates significance ($p<0.05$).

	A) MALES				B) FEMALES			
	Transgenic		**Normal**		**Transgenic**		**Normal**	
LD	Mass	S.D.	Mass	S.D.	Mass	S.D.	Mass	S.D.
Gonads	0.873	0.398	0.619	0.511	0.618	0.725	0.858	0.553
Adrenals	0.201	0.197	0.076	0.078	0.313	0.365	0.208	0.204
Kidneys	5.616	3.382	3.251	2.405	3.935	3.935	3.334	2.355
Females	Mass	S.D.	Mass	S.D.	Mass	S.D.	Mass	S.D.
Gonads	0.798	1.127	0.858	0.553	0.990	1.034	0.396	0.285
Adrenals	0.171	0.111	0.208	0.204	0.743	0.811	0.260	0.176
Kidneys	4.566	4.275	3.334	2.355	6.057*	4.938	1.828*	1.505

TABLE 2.8. Comparison of right and left organ mass difference (mg) in A) transgenic and B) normal males versus females in 12h dark:12h light and 24h light photoperiods. (*) indicates significance ($p<0.05$).

	A) TRANSGENIC				B) NORMAL			
	Males		**Females**		**Males**		**Females**	
LD	Mass	S.D.	Mass	S.D.	Mass	S.D.	Mass	S.D.
Adrenals	0.201	0.197	0.313	0.365	0.076*	0.078	0.303*	0.264
Kidneys	5.616	3.382	3.935	2.784	3.251	2.405	3.692	2.600
LL	Mass	S.D.	Mass	S.D.	Mass	S.D.	Mass	S.D.
Adrenals	0.171*	0.111	0.743*	0.811	0.208	0.204	0.260	0.176
Kidneys	4.566	4.275	6.057	4.938	3.334	2.355	1.828	1.505

TABLE 2.9. Comparison of absolute right and left organ mass difference (mg) in 12hr light:12hr dark versus 24hr light photoperiods in A) transgenic and B) normal males and females. (*) indicates significance ($p<0.05$).

	A) TRANSGENIC				B) NORMAL			
	LD		LL		LD		LL	
Males	Mass	S.D.	Mass	S.D.	Mass	S.D.	Mass	S.D.
Gonads	0.873	0.398	0.798	1.127	0.619	0.511	0.858	0.553
Adrenals	0.201	0.197	0.171	0.111	0.076	0.078	0.208	0.204
Kidneys	5.616	3.382	4.566	4.275	3.251	2.405	3.334	2.355
Females	Mass	S.D.	Mass	S.D.	Mass	S.D.	Mass	S.D.
Gonads	0.618	0.725	0.990	1.034	0.459	0.369	0.396	0.285
Adrenals	0.313*	0.365	0.743*	0.811	0.303	0.264	0.260	0.176
Kidneys	3.935	2.784	6.057	4.938	3.692*	2.600	1.828*	1.505

SECTION III

FEEDING BEHAVIOUR IN GIANT TRANSGENIC RAT GROWTH HORMONE MICE: IMPACTS OF SEX AND CONSTANT ILLUMINATION

3

3.1 RATIONALE AND OBJECTIVES

This thesis explores how feeding, reproduction and activity are altered in mice expressing mulitiple copies of rat growth hormone genes. It also demonstrates differences between a 12:12 (LD) photoperiod versus constant light (LL). The main objective of this study was to examine the alteration of transgenic rat GH mice (TRrGHm) relevent to their reproductive impairment. This required monitoring of i) life-history features (body size, fecundity, organ sizes), ii) feeding rates, and iii) behavioural time budgets (sleeping, locomotion, wheel running, feeding, drinking).

This paper focuses on the second component of organism design, specifically the impact of excess growth and continuous light on feeding behaviour. In addition, feeding rates of female normal and TRrGHm have not previously been published. The feeding rates of transgenic GLUT-4 mice which contain double the amount of GLUT-4 insulin responsive glucose transporters was also assessed. This was performed in the hope that GLUT-4 mice, having improved glucose uptake, might show elevated feeding . If so, GH-GLUT-4 hybrids might have elevated feeding that would alleviate the state of energetic stress detected in GH transgenics. For example, this might restore reproduction by allowing greater allocation of resources to their function despite accelerated growth. We also intended to compare feeding rates between males and females to determine if females were compensating for their increased reproductive demands by eating more.

3.2 CLARIFICATION OF CONTRIBUTIONS

This study was proposed by C. David Rollo, as a component of a larger research endeavor examining altered allocation and functions in TRrGHm. The current study examined the impact of constant light on reproduction, organ allometries, feeding behaviour, and activity of TRrGHm and normal controls of both sexes.

My contributions were as follows:

1. **Intellectual Contributions:**

   (i) Review of scientific literature on transgenic GH and GLUT-4 animals, and neuroendocrinological data was conducted largely by myself.

   (ii) The theories and ideas presented in this thesis were synthesized jointly by Dr. C.D. Rollo and myself.

2. **Standard Animal Care of Main Breeding Colonies:**

   All aspects of animal care were performed in conjunction with Dr. L. Kajiura.

   Status of Health

   (i) The status of health was checked for all animals daily. Cages were inspected for dead animals. Identification numbers, sex, birthdate, date of death, coat colour, strain (transgenic or normal), and diet treatment were noted for the deceased. I was specifically responsible for culling the colony, and euthanizing any ill or injured mice.

   (ii) Cages were checked daily for pregnant females and new litters. Identification numbers of parents, sex, coat colours, strain (transgenic or normal), birthdate of offspring, and offspring body mass were recorded to complile data on the

reproduction for transgenic and normal strains. At 20 days of age, mice were weaned to form new breeding groups or to be used for experimental purposes.

Food

Cage hoppers were stocked daily with standard rodent food pellets (LabDiet®, No. 5001, PMI Feed Inc., APPENDIX A) provided *ad libitum.*

Water

Water bottle were topped up daily. All bottles were washed with detergent and sterilized at three-day intervals.

Animal Breeding Cages

Cages (length x width x height = 28 x 17.5 x 5 cm), which contained 1 male and 4 females were washed, disinfected, and bedding material (wood chips) were replaced every 3 days.

3. Maintenance of Animal Quarters:

Floors, holding racks, and bench tops were washed and disinfected daily. Room temperature and photoperiod (L:D 12h:12h; L:L 24h) were also checked each day.

4. Experimental Design, Preparation, Data Documentation, and Statistical Analyses for Feeding Study:

(i) Preparation, construction, and assembly of experimental feeding containers.

(ii) Preparation, assembly, and weighing of food pellet dishes for daily

measurements of food consumption..

(iii) Documentation and calculation of food consumption. Statistical comparisons (regression analyses, ANCOVA) were conducted using JUMP and MINITAB.

3.3

Feeding behaviour in giant transgenic rat growth hormone mice: impacts of sex and constant illumination.

by

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Extension 22574

3.4 ABSTRACT

Transgenic rat growth hormone mice (TRrGHm) possess multiple copies of rat growth hormone genes and grow to double normal sizes at double normal rates. GLUT-4 mice possess 5 to 7 copies of an insulin responsive glucose transporter gene which elevates glucose uptake into tissues. To determine how feeding rates are altered by these transgenic strains, a study was conducted on 120 normal, TRrGH, and GLUT-4 males and female mice in 12h dark:12h light (LD). These were compared to 80 normal and TRrGH males and females in 24h light (LL) to determine how photoperiod affects feeding (GLUT-4 mice were only observed in LD). The mean dry body mass of TRrGH males was 207% that of normal males. TRrGH females were 164% heavier than normal females. Normal males weighed 144% more than GLUT-4 males, and the dry body mass of normal females was 128% higher than GLUT-4 females. Photoperiod did not significantly affect body mass. TRrGH males and females mass-specifically consumed less food than normal males and females respectively in both LD and LL. This suggests that enhanced growth of TRrGHm did not occur by increased energy intake, but rather resources were diverted from other processes such as reproduction, behaviour, or longevity. Evidence for such trade-offs supports the "principle of allocation," a key assumption for theories of life-history evolution. GLUT-4 males and females also ate less than their normal counterparts, a result that was not anticipated since they have the capacity for increased clearance of plasma glucose. In normal and TRrGHm, males ate less than females in both light treatments suggesting that gonadal hormones played a major role in feeding independent of the GH axis. Feeding did not significantly differ in either normal or TRrGH males between LD and LL. TRrGH females, however, showed a marked reduction in feeding in LL suggestive of altered estrogen levels.

3.5 INTRODUCTION

Animals have limited ability to process and acquire resources which must be allocated among conflicting demands of growth, reproduction, maintenance, defense, storage, and behaviour. Enhancement of one attribute must negatively impact on others as a result of energetic limitation. Such trade-offs are the basis of the "principle of allocation" that underlies much of life-history theory (Rollo 1994). Transgenic animals are useful in testing the principle of allocation. Transgenisis amplifies one feature and disrupts its coadaptation with others. This is ideal for understanding the integration of processes constituting a species' adaptive suite.

TRrGHm contain multiple copies of rat growth hormone fusion genes incorporated into one chromosome. This results in growth rates and body sizes twice that of normal mice (Palmiter *et al.* 1982). TRrGH males did not increase mass-specific feeding to compensate for increased growth (Kajiura and Rollo, 1994), so negative trade-offs with other requirements are predicted. One consequence of accelerated growth has been observed in TRrGH females which exhibit reproductive deficiencies. Experiments with transgenic human and bovine GH mice indicate that this occurs as a result of luteal failure associated with loss of mating-induced prolactin surges (Bartke *et al.* 1988) (see Section II).

GLUT-4 mice, another transgenic strain, exhibit 2-fold the amount of insulin responsive glucose transporters (Ezaki *et al.* 1993), and greatly enhanced peripheral glucose transport (Treadway *et al.* 1994). Glucose is believed to be a key regulatory signal for feeding regulation. Our purpose was to evaluate the physiological impacts of enhanced growth and increased tissue glucose uptake on the feeding of TRrGH and GLUT-4 transgenic males and females. This study is one component of a larger effort that examines the impact of transgenesis and environmental alterations on reproduction, feeding, and

behavioural time budgets of mice. Specifically, we examine the impact of continuous light on feeding behaviour in TRrGH mice, as well as examine normal feeding patterns of GLUT-4 mice in normal 12h dark;12h light (LD) photoperiod. In addition, female feeding behaviour in both strains of transgenic mice is examined to assess the impact of increased reproductive costs on feeding. It is our hope to eventually cross-breed GH-GLUT-4 transgenic mice. TRrGHm have chronic insulin resistance (reduced peripheral glucose transport), that could impact plasma glucose turnover and underly their hypophagia (Bartke *et al.* 1994). The GLUT-4 transgenes were examined with the idea of reducing this peripheral insulin resistance and thereby restoring normal glucose utilization and feeding. We hypothesize that the resulting offspring would be able to compensate for the energetic demands of accelerated growth through improved glucose utilization. Through this it may then be possible to improve or abolish TRrGH female infertility.

3.6 METHODS

3.6.1 *Animals Used*

Transgenic mice are engineered by microinjection of genes into the pronuclei of fertilized mouse eggs. These eggs are subsequently implanted into pseudopregnant foster mothers. "Supermice" (*Mus musculus*, transgenic strain Tg[MT-1,rGH]Bri2) were created by Palmiter *et al.* (1982) via microinjection of rat GH structural genes fused to metallothionein-1 promoters. These mice have multiple copies of rGH genes incorporated into one chromosome, resulting in plasma GH levels being elevated to 100-400 times normal levels (Palmiter *et al.* 1982; Shea *et al.* 1987). GH mosulates growth via insulin-like growth factors (somatomedins) that activate receptors in target tissues (Matthews *et al.* 1988).

GLUT-4 mice (background strain C5BL/KsJ-mt/mt) were engineered by Ezaki *et al.* (1993) through microinjection of a GLUT-4 minigene containing: 7kb of the 5' flanking and 1kb of the 3' flanking sequence, all the introns and exons of the GLUT-4 gene and a small foreign DNA tag. This resulted in 5 to 7 copies of the minigene incorporated into one chromosome. The GLUT-4 protein is an insulin responsive transporter responsible for glucose uptake into insulin regulated tissues. Transgenic GLUT-4 mice showed less than or equal to a 2-fold increase in the GLUT-4 protein, but this resulted in a very rapid blood glucose clearance rate. Inheritance of both the rGH genes and GLUT-4 genes is Mendelian.

Of the 200 animals studies, 40 were homozygously transgenic for the GLUT-4 minigene, 80 were heterozygously transgenic for the rGH gene, and 80 lacked transgenes and served as normal controls. The GLUT-4 transgenics and the TRrGHm are from different background strains, however, and final publication will require appropriate strain controls. However, the present results are sufficient to demonstrate that a significant

elevation in feeding in the GLUT-4 mice is unlikely to be realized. Transgenic rGH animals were easily differentiated by their larger size at 28 days of age. Ten groups were examined, 6 in 12h dark:12h light (LD) and 4 in 24h light (LL) photoperiods. Each light treatment consisted of: 20 normal males, 20 normal females, 20 transgenic rGH males and 20 transgenic rGH females. 20 GLUT-4 males and 20 GLUT-4 females were examined in LD only.

3.6.2 *General Rearing Conditions*

Breeding groups (1 male mated to 4 females) were housed in clear plastic cages (length x width x height = 28 x 17.5 x 5 cm) at 22 +/- 2° C with a photoperiod of either 24h light or 12h dark:12h light. A stainless steel hopper, placed over each container supported food pellets and a water bottle. All animals were fed *ad libitum* (Lab Diet®, No. 5001, PMI Feed Inc.). Cages were bedded with BetaChips® (Hardwood Laboratory Bedding) and cleaned every 3 days. Each cage contained an enrichment bottle or plastic tube. All protocols were consistent with the guidelines of the Canadian Council on Animal Care.

3.6.3 *Feeding Rate Containers*

Mice were preweighed and placed individually in cages modified to measure feeding rates. Regular breeding cages were modified as follows. (I) Bedding was removed and replaced by a raised steel grid floor (7-mm mesh). (II) Food was removed from the hopper, and 11 preweighed pellets were glued to the bottom of a 5.3 cm diameter plastic petri dish with RTV 108 Silicone Rubber Adhesive Sealant, GE Canada Inc®. Each dish was fastened within a 9 cm diameter plastic petri dish using tape, and placed on the grid floor. This prevented food transport and spillage. Food dishes were prepared 2 weeks prior to use to ensure that the silicone was fully dried. Food dishes were replaced before the mice could encounter the silicone.

3.6.4 *Variables Investigated*

Feeding rates for each mouse were performed over 10 consectutive days (variety of ages) in both LD and LL. Every other day data were recorded, cages were cleaned and the food and water replaced. Mice were weighed on the first and last day of each experiment. Ten transgenic rGH and 10 normal mice were sacrificed and oven-dried to constant mass at 60 C to provide wet-mass to dry-mass conversion factors. Water content did not vary across types of mice but did vary with age (see Appendix B). All values reported are in dry mass units.

Data was recorded every other day by first collecting any partially consumed food pellets and crumbs. These were then weighed and replaced. Consumption was calculated by subtracting the dried mass of uneaten food from the estimated dry mass provided initially.

The following variables were considered: dry mass of food consumed (mg), dry mouse body mass (g). Individual rates, expressed in grams food per grams dry body mass, were calculated as averages of the 10-day period and then converted to daily measures.

3.6.5 *Analytical Methods*

Feeding rates were calculated in grams dry mass per gram dry body mass per day to eliminate body size differences between normal, transgenic rGH and GLUT-4 animals. Overall means (+/-S.D.) were calculated for each variable. To facilitate comparisons transgenic rGH and GLUT-4 results were also expressed as a percentage of respective normal values. Statistical analysis consisted of regression analysis and analysis of covariance (ANCOVA).

3.7 RESULTS

Feeding rates for normal, TRrGH, and GLUT-4 mice are provided in Figures 3.1 to 3.7 and reveal the impact of photoperiod, growth enhancement, and increased glucose uptake on feeding.

3.7.1 *Normal and TRrGH Mice in 12h dark:12h light (LD)*

TRrGH males ate significantly less (74%) than TRrGH females in LD (0.489 +/- 0.101 versus 0.659+/- 0.148 g/g dry body mass respectively, $p<0.000$). Normal males also ate less (94%) than their female counterparts (0.702 +/- 0.144 versus 0.744 +/- 0.174 g/g dry body mass respectively, $p<0.01$). Normal males ate 144% more than TRrGH males (0.702 +/- 0.144 versus 0.489 +/- 0.101 g/g dry body mass respectively, $p<0.000$), whereas normal females ate only 113% more than TRrGH females (0.744 +/- 0.174 versus 0.659 +/- 0.148 g/g dry body mass respectively, $p<0.001$, Figure 3.1).

3.7.2 *Normal and TRrGH Mice in 24h light (LL)*

TRrGH males ate significantly less (79%) than normal males in LL (0.573 +/- 0.119 versus 0.720 +/- 0.131 g/g dry body mass respectively, $p<0.002$), and TRrGH females also ate less (75%) than normal females (0.562 +/- 0.118 versus 0.752 +/- 0.131 g/g dry body mass respectively, $p<0.000$) In this treatment significant differences in feeding between males and females disappeared in both kinds of mice (Figure 3.2).

3.7.3 *Normal and TRrGH Females in LD versus LL*

TRrGH females in LD ate 117% more than in LL (0.659 +/- 0.148 versus 0.562 +/- 0.118 g/g dry body mass respectively, $p<0.003$). Normal females did not differ significantly between light treatments (Figure 3.3).

3.7.4 *Normal and TRrGH Males in LD versus LL*

TRrGH males in LL ate 117% more than those in LD (0.573 +/- 0.119 versus 0.489 +/- 0.101 g/g dry body mass respectively, $p<0.02$). Normal males did not differ significantly between LD and LL (Figure 3.4).

3.7.5 *Normal, TRrGH, and GLUT-4 Females in LD*

Normal females ate 128% more than GLUT-4 females (0.744 +/- 0.174 versus 0.581 +/- 0.194 g/g dry body mass respectively, $p<0.008$). GLUT-4 and TRrGH females did not differ significantly in feeding (Figure 3.5).

3.7.6 *Normal, TRrGH, and GLUT-4 Males in LD*

GLUT-4 males ate significantly less than normals (69.5%) in LD (0.488 +/- 0.126 versus 0.702 +/- 0.144 g/g dry body mass respectively, $p<0.000$), but no difference was observed between GLUT-4 and TRrGH males (Figure 3.6).

3.7.7 *GLUT-4 Males and Females in LD*

No significant difference in feeding was observed between male and female GLUT-4 mice in LD. GLUT-4 mice were not examined in LL.

3.8 DISCUSSION

Many functions of TRrGHm are energetically limited due to excessive allocation of resources into growth (i.e. tradeoffs according to the principle of allocation). There are several neuroendocrinological consequences. Persistant ectopic production of GH and elevation of peripheral GH leads to suppression of endogenous GH release and pituitary atrophy (Sotelo *et al.* 1993). This occurs since somatostatin (SRIF), a GH inhibitor, is elevated (Hurley *et al.* 1992) and GHRH (GH releaser) (Krulich *et al.* 1968), is depressed (Bartke *et al.* 1994). Feeding may be affected by upregulation of SRIF. Starved rats eat less when administered SRIF (Hugues *et al.* 1986). The present study clearly demonstrated that TRrGH mice ate less on a mass-specific basis than their non-transgenic siblings in both LD and LL. It is therefore probable that the elevated SRIF levels observed in TRrGH mice negatively impacted on their feeding behaviour.

TRrGHm are hypoprolactinemic (Bartke *et al.* 1988; Chandreshekar *et al,* 1992), and the mating-induced surges of PRL are absent (Cecim *et al.* 1995). This results from an abnormal pattern of changes in the activity of tuberofundibular dopaminergic (DA) neurons. Normally, DA activity decreases during PRL surges, but in TRrGHm activity levels remain elevated. DA inhibits feeding in starved rats (Leibowitz, 1980, 1978). Since DA activity is abnormal in TRrGHm, elevated DA could contribute to the hypophagia in TRrGHm. DA could act directly or through stimulation of SRIF release (Chihara *et al.* 1979; Kitajima *et al.* 1989).

A possible cause of reduced mass-specific feeding in TRrGHm is elevation of the hypothalamic-pituitary-adrenal (HPA). Plasma levels of corticosterone are significantly elevated under both basal and stress conditions (Cecim *et al.* 1991). This appears to be due to increased adrenocorticotropic hormone (ACTH) secretion rather than direct effects of GH or insulin-like growth factor 1 (IGF-1) on the adrenal cortex (Bartke *et al.* 1994). It is

suspected that this results from an elevation of corticotropin releasing factor (CRF) drive to the pituitary. CRF significantly downregulates feeding in starved rats (Morley and Levine 1982; Krahn *et al.* 1988; Hotta *et al.* 1991). CRF elevates levels of both beta-endorphin (Rivier*et al.* 1982) and norepinephrine (NE) (Brown *et al.* 1982). In a study by Morley and Levine (1980) it was demonstrated that beta-endorphin decreases feeding in the light. Similarly, NE can also regulate feeding. Whether NE positively or negatively impacts on feeding behaviour is dependent upon the type of adrenergic receptor activated. In the paraventricular nucleus (PVN), NE injections stimulate feeding behaviour in satiated rats via alpha2-adrenergic receptors (Leibowitz 1970, 1981; Marino *et al.* 1983). The magnitude of alpha2-NE mediated feeding varies directly with levels of circulating corticosterone (Bhakthavatsalam and Leibowitz, 1986; Jhanwar-Uniyal *et al.* 1986). NE injections into the lateral hypothalamus inhibits feeding in hungry rats via beta-adrenergic receptors (Leibowitz 1970, 1981; Leibowitz and Rossakis 1978; Krahn *et al.* 1988). Beta receptors also downregulate endogenous GH production (Muller *et al.* 1993) TRrGHm may exhibit increased beta-receptor, or reduced alpha2-NE binding, since other mechanisms downregulating endogenous GH production are elevated in TRrGHm with chronically elevated exogenous GH. The elevated HPA axis could also impact on feeding via increased levels of DA, since stress elevates DA in rats (Imperato *et al.* 1989; Abercrombie *et al.* 1989).

Ovarian hormones alter feeding in female rats and could also have affected the feeding behaviour in the TRrGH females. TRGHm exhibit reduced progesterone levels (Cecim *et al.* 1995), and progesterone has been shown to increase feeding in the presence of estrogen (Gray and Wade, 1981). In addition, estrogen decreases food intake in rats (Wade and Gray, 1978; Gray and Wade, 1981; Ramirez, 1981; Butera and Beikirch, 1988; Sarkar *et al.* 1989) and IGF-1, which is elevated in TRrGHm, promotes estrogen production (Adashi 1994). There are various ways in which estrogen may inhibit feeding behaviour.

Estrogen elevates the HPA axis in female rats (Burgess and Handa, 1992; Carey *et al.* 1995). Burgess and Handa (1992) demonstrated elevated ACTH and corticosterone in response to estrogen due to impaired glucocorticoid receptor-mediated slow negative feedback on the HPA axis. Also, estrogen increases serotonin receptor binding in the hypothalamus (Butera and Beikirch 1988). This is consistent with the hypothesis that the changes in food intake produced by estrogens are mediated by increased serotonergic activity. The neuroactive peptide cholecystokinin (CCK) is also involved in feeding regulation (Gibbs and Smith 1986), and estrogens influence the activity of CCK (Akesson *et al.* 1987; Oro *et al.* 1988). The possibility that estrogenic suppression of food intake is mediated by changes in CCK remains to be investigated.

This study demonstrated that males ate less than females (both TRrGH and normal mice) in both light treatments. One reason may be that progesterone levels in females are higher and, as stated earlier, progesterone increases feeding behaviour (Gray and Wade 1981). Another possibility is that males have high testosterone levels. Wade and Gray (1978) demonstrated that testosterone decreases feeding and adiposity in male rats. A final reason as to why females eat more than males is simply because females require increased energy supplies to compensate for their increased reproductive demands.

TRrGH females ate significantly less in LL than LD. There are two reasons as to why this may have occured. Beta-endorphin reduces feeding during the light phase (Morley and Levine 1980). Due to the absence of darkness, beta-endorphin may have exerted a greater impact on the the downregulation of feeding in LL. In addition, NE reduces feeding by binding to beta-adrenergic receptors and this binding shows circadian rhythmicity (Krauchi *et al.* 1984). Beta-adrenergic receptor binding is reduced, and alpha2 receptor binding increases after dusk, allowing for increased feeding. In LL, this circadian pattern may have been compromised allowing for prolonged binding to beta-adrenergic receptors, thereby further reducing feeding. These reasons, in conjunction with the neuroendocrine

abnormalities already present in TRrGH females, may have played a role in the reduced feeding pattern observed in LL.

GLUT-4 mice eat less mass-specifically than normal non-transgenic mice in LD. This could be a result of improved energy utilization in these mice. GLUT-4 mice contain double the amount of the GLUT-4 transporter (Ezaki *et al.* 1993), an insulin responsive glucose transporter responsible for glucose uptake into peripheral tissues like adipose and muscle tissue (Kaestner *et al.* 1986; James *et al.* 1989; Charron *et al.* 1989; Birnbaum 1989; Fukumoto *et al.* 1989). Due to the rapid blood clearance rate of glucose observed in these mice (Ikemoto *et al.* 1994), it is possible that less food needs to be ingested to have the same amount of glucose uptake into the tissues. Plasma glucose is one of the major factors suggested to contribute to feeding regulation. Reduced feeding in conjunction with upregulated transport efficiency suggests that tissue uptake may be the key signal rather than plasma titers (i.e. the sensor is intracellular and not tuned to extracellular conditions *per se).* Leturque *et al.* (1996) demonstrated that GLUT-4 mice also have increased insulin responsiveness. Insulin is responsible for stimulating translocation of the GLUT-4 transporter to the plasma membrane for glucose uptake. These two factors would suggest that GLUT-4 mice would have an increased efficiency in energy utilization, however this has yet to be investigated.

In conclusion, TRrGH mice eat less than their non-transgenic siblings. This could result from increased SRIF, DA, or HPA axis hormones. The increased activity of the HPA axis may impact on both NE-induced inhibition of feeding via beta-adrenergic receptors, as well as increased beta-endorphin release. In females, elevated estrogen and decreased progesterone levels may have also contributed to downregulation of feeding behaviour. The fact that TRrGH females ate less in LL could be attributed to increased binding of NE to beta-adrenergic receptors as well as upregulation of beta-endorphin. This would likely contribute to energetic shortfalls in allocation to reproduction, consequently

accentuating infertility. Testosterone reduces feeding and may have been involved in the decreased feeding observed in males. Increased efficiency in energy utilization could have been the underlying source of reduced feeding observed in transgenic GLUT-4 mice although this has yet to be examined.

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FIGURE 3.1. Feeding rate of normal males (- - -), transgenic males (— —), normal females (- - -), and transgenic females (——) in 12h dark:12h light (LD) photoperiod. Transgenic males and females ate significantly less than normal males and females respectively ($p<0.000$, $p<0.001$). Transgenic males ate significantly less than transgenic females ($p<0.000$) and normal males ate less than normal females ($p<0.013$).

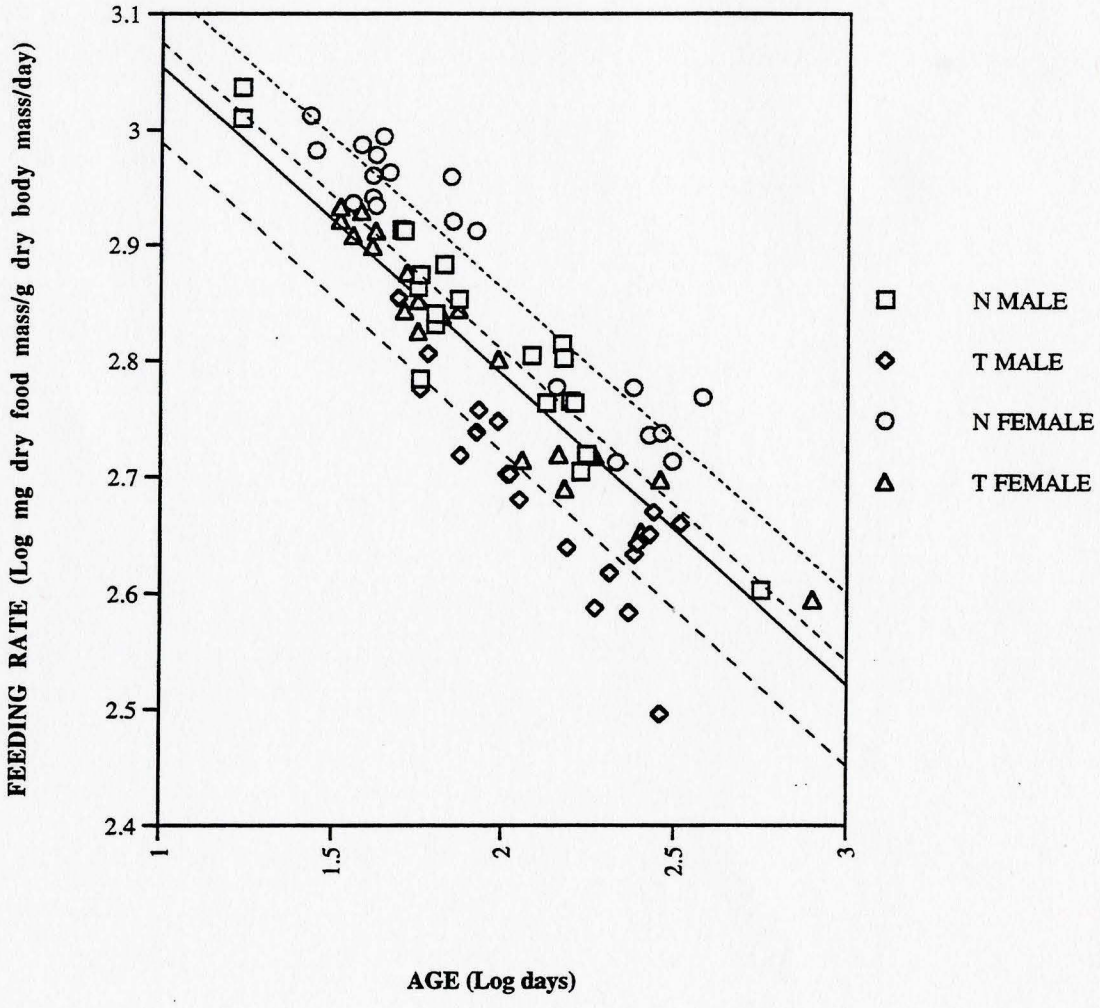

FEEDING RATE (Log mg dry food mass/g dry body mass/day)
3.1
3
2.9
2.8
2.7
2.6
2.5
2.4
1
1.5
2
2.5
3
AGE (Log days)
N MALE
T MALE
N FEMALE
T FEMALE

FIGURE 3.2. Feeding rate of normal males (- -), transgenic males (— —), normal females (- - -), and transgenic females (——) in 24 light (LL) photoperiod. Transgenic males and females ate significantly less than normal males and females respectively ($p<0.002$, $p<0.000$). There were no significant differences in feeding between male and female transgenics and normals.

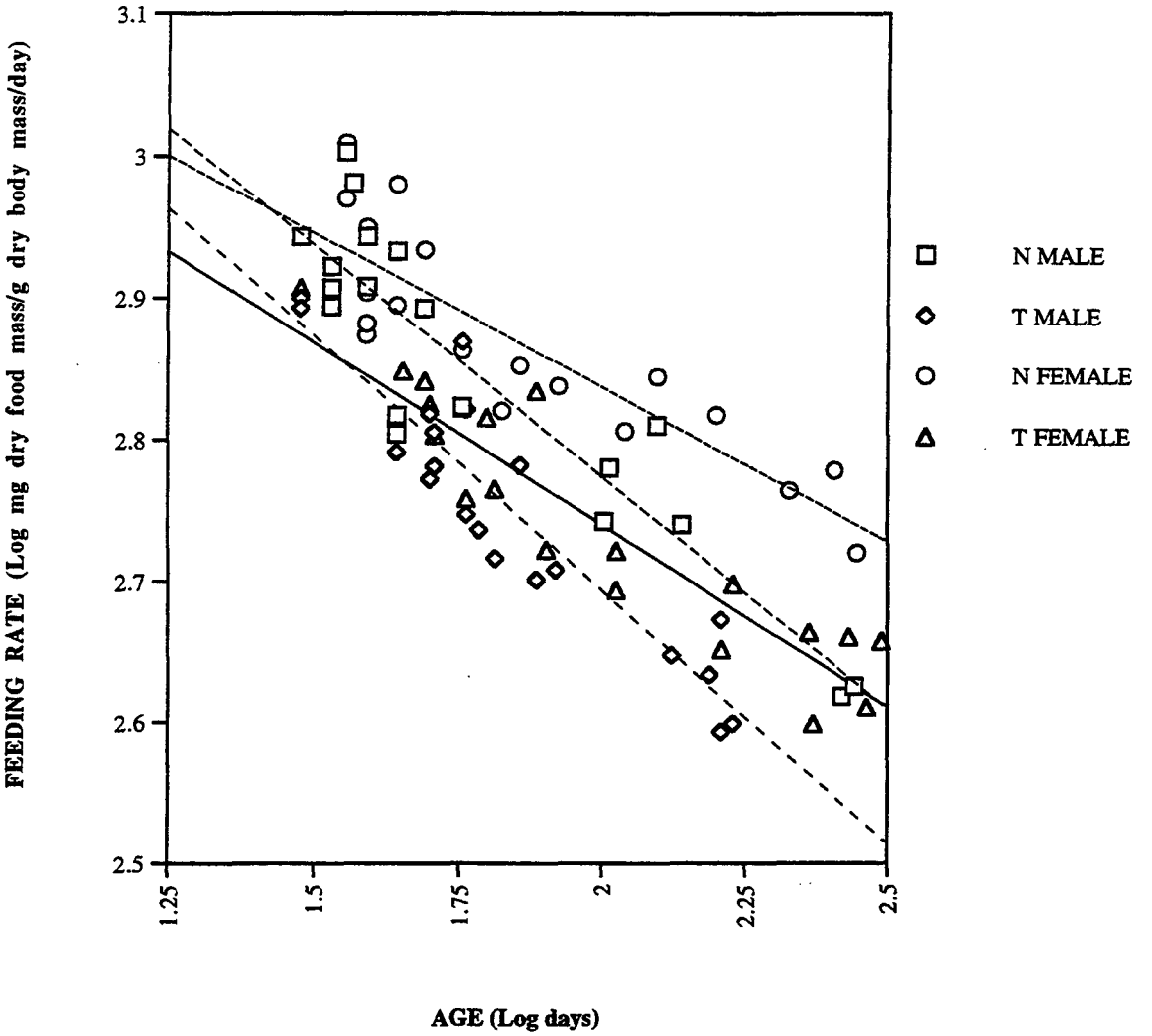

FEEDING RATE (Log mg dry food mass/g dry body mass/day)
3.1
3
2.9
2.8
2.7
2.6
2.5
1.25
1.5
1.75
2
2.25
2.5
AGE (Log days)
N MALE
T MALE
N FEMALE
T FEMALE

FIGURE 3.3. Feeding rate of normal females in 12h dark:12h light (LD) (- - -) and 24h light (LL) (— —) and transgenic females in LD (- - -) and LL (——) photoperiods. Transgenic females in LL ate less ($p<0.003$). Normal females did not differ significantly. Transgenic females ate significantly less than normals ($p<0.001$, $p<0.000$) in both LD and LL respectively.

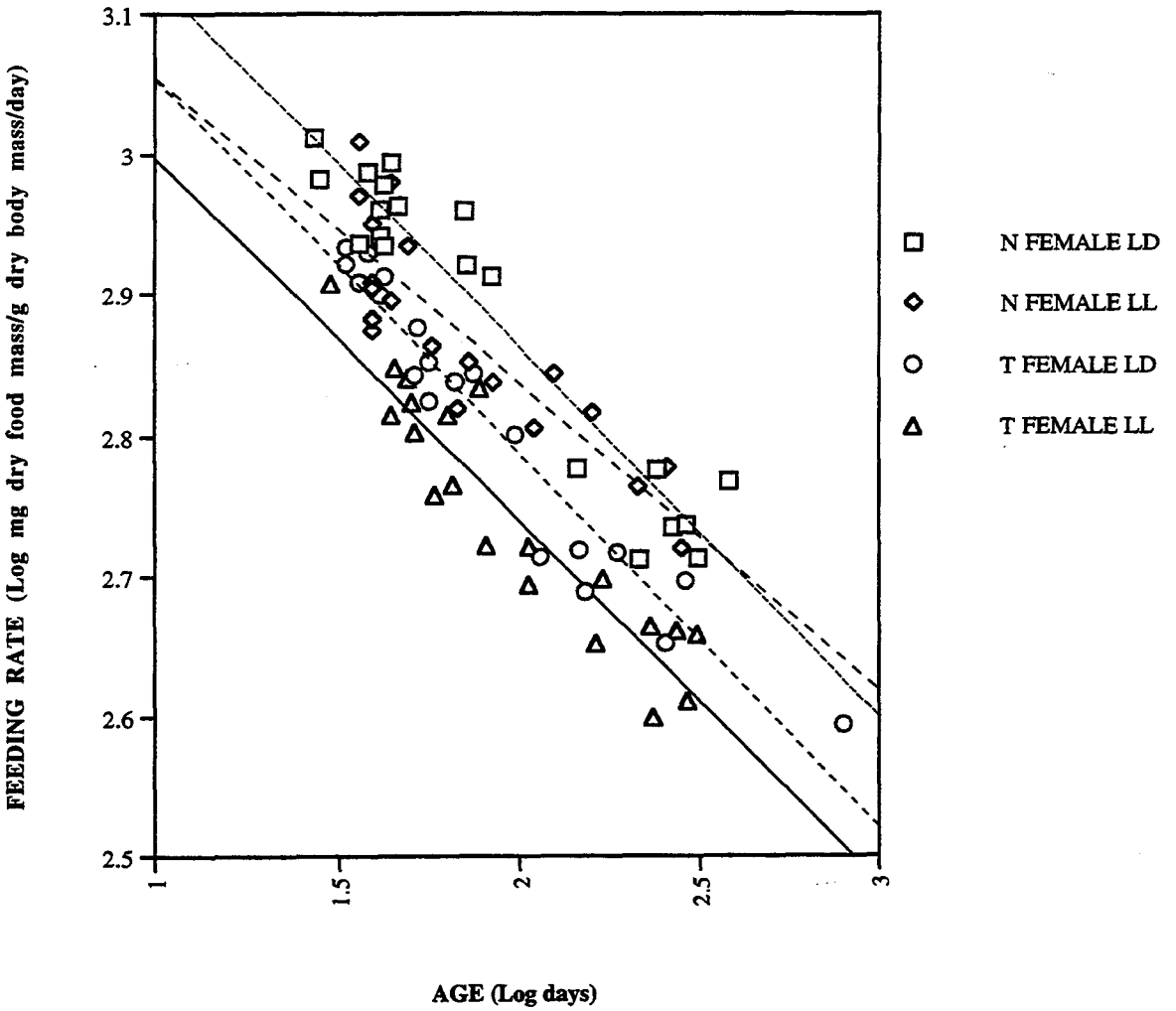

FEEDING RATE (Log mg dry food mass/g dry body mass/day)
3.1
3
2.9
2.8
2.7
2.6
2.5
1
1.5
2
2.5
3
AGE (Log days)
N FEMALE LD
N FEMALE LL
T FEMALE LD
T FEMALE LL

FIGURE 3.4. Feeding rate of normal males in 12h dark:12h light (LD) (- -) and 24h light (LL) (- - -) and transgenic males in LD (—) and LL (- - -) photoperiods. Transgenic males in LL ate more ($p<0.022$). Normal males did not differ significantly. Transgenic males ate significantly less than normals ($p<0.000$, $p<0.002$) in both LD and LL respectively.

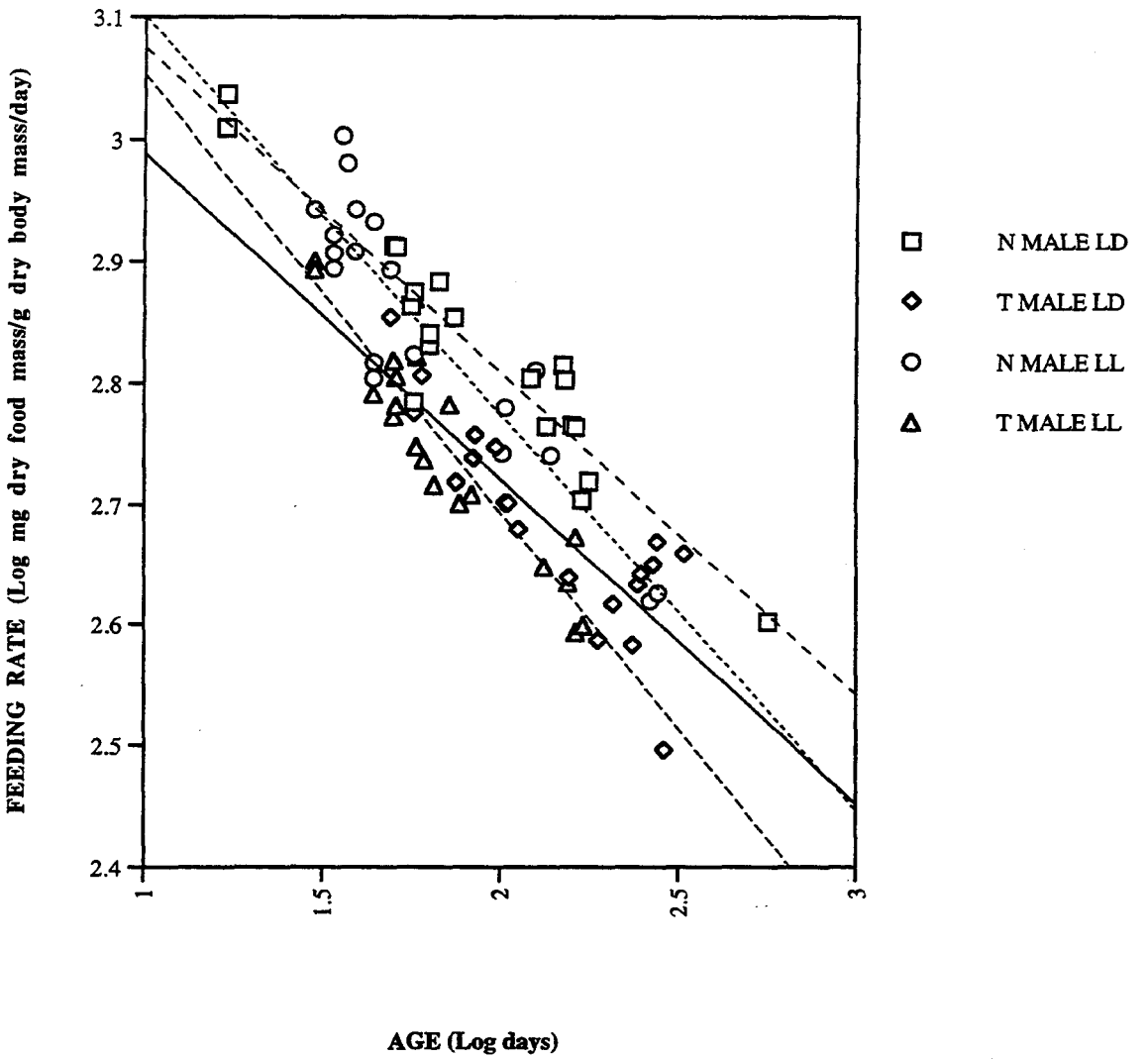

FEEDING RATE (Log mg dry food mass/g dry body mass/day)
3.1
3
2.9
2.8
2.7
2.6
2.5
2.4
1
1.5
2
2.5
3
AGE (Log days)
N MALE LD
T MALE LD
N MALE LL
T MALE LL

FIGURE 3.5. Feeding rate of normal (- - -), transgenic (——), and glut-4 (– –) females in 12h dark:12h light (LD). Transgenic females ate significantly less than normals ($p<0.001$). There was a significant difference in feeding between glut-4 and normal females ($p<0.008$) but not with the transgenics.

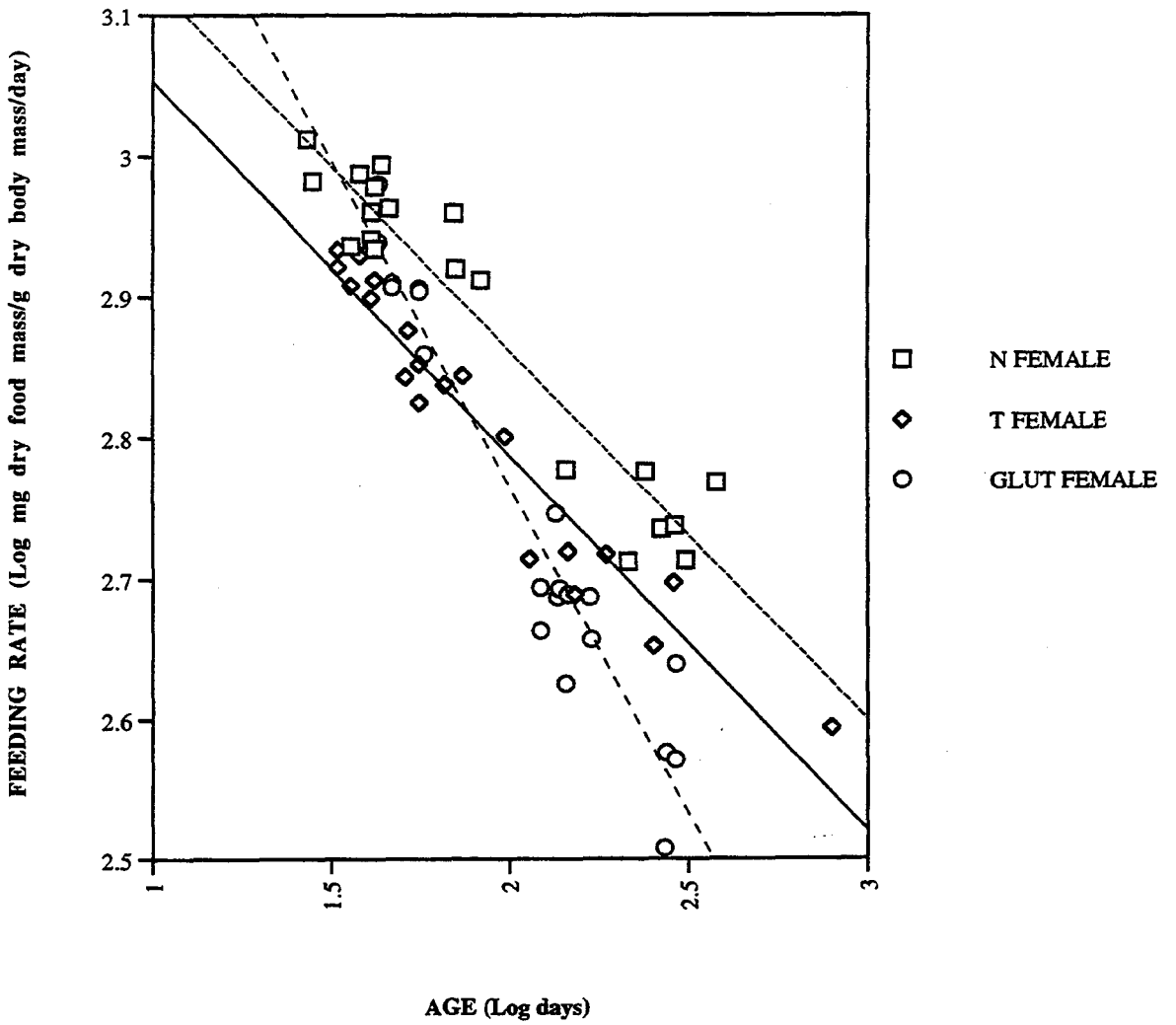

FEEDING RATE (Log mg dry food mass/g dry body mass/day)
3.1
3
2.9
2.8
2.7
2.6
2.5
1
1.5
2
2.5
3
AGE (Log days)
N FEMALE
T FEMALE
GLUT FEMALE

FIGURE 3.6. Feeding rate of normal (---), transgenic (—), and glut-4 (–-) males in 12h dark:12h light (LD). Transgenic males ate significantly less than normals ($p<0.000$). There was a significant difference in feeding between glut-4 and normal males ($p<0.000$) but not with the transgenics.

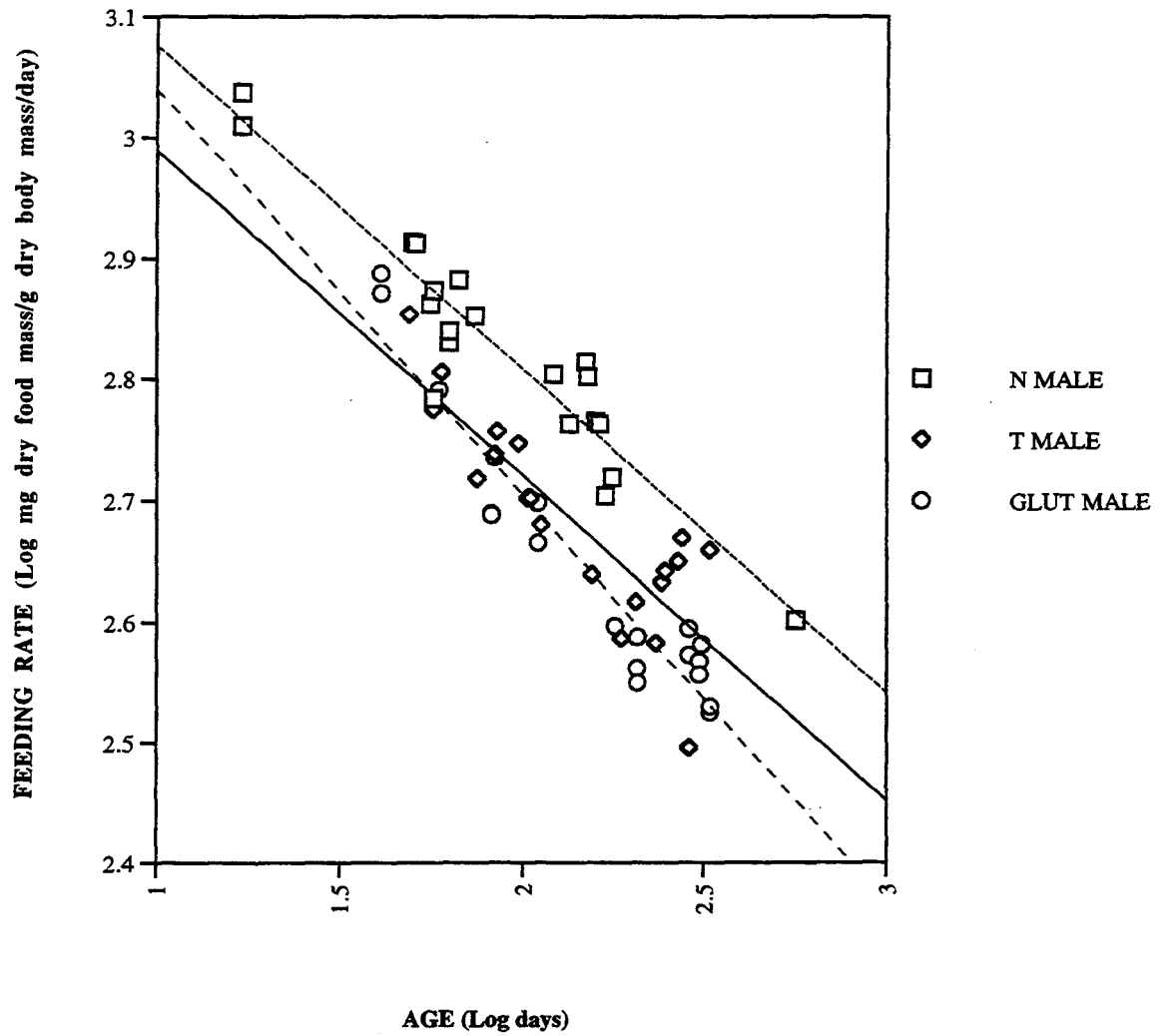
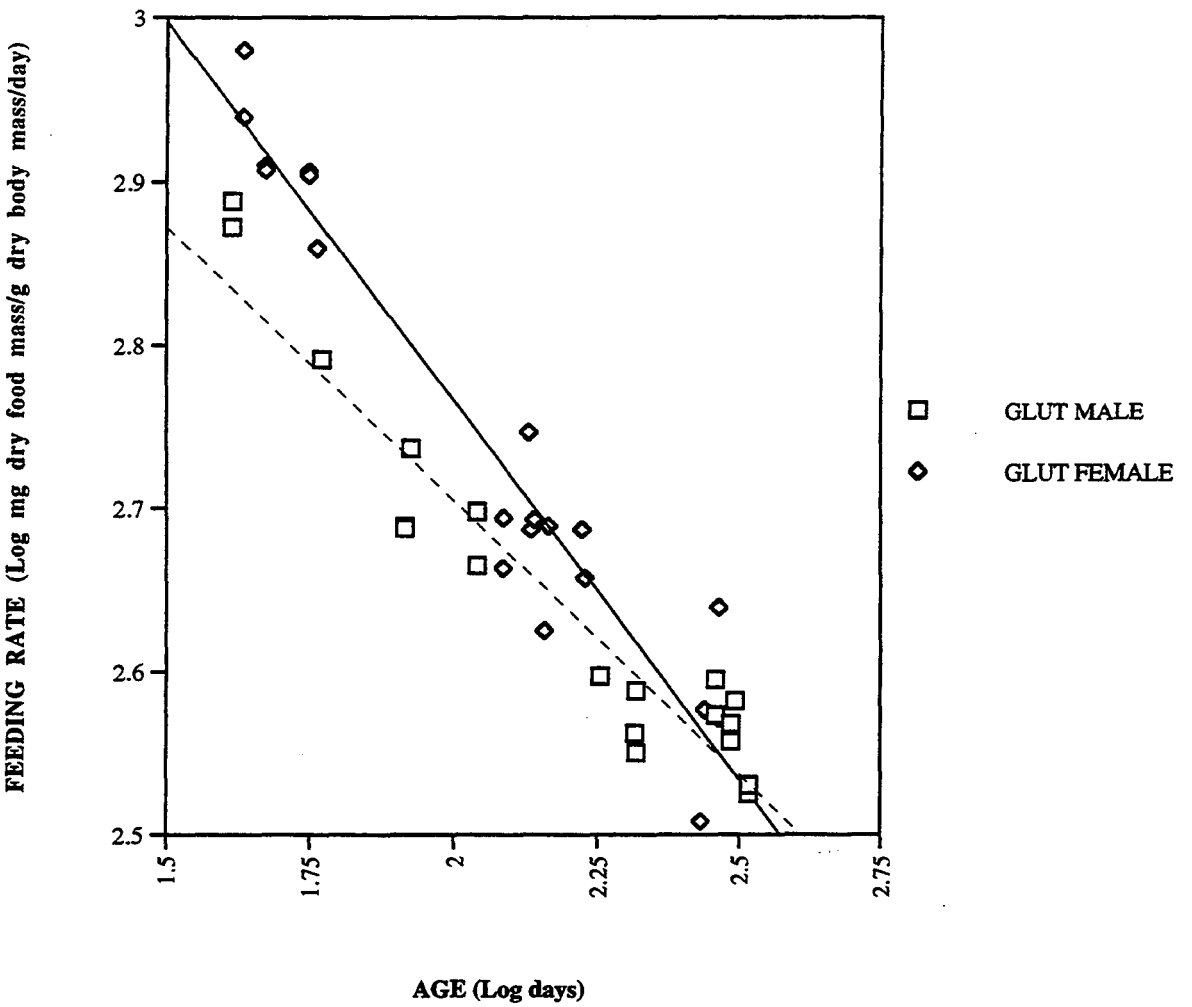

FEEDING RATE (Log mg dry food mass/g dry body mass/day)
3.1
3
2.9
2.8
2.7
2.6
2.5
2.4
1
1.5
2
2.5
3
AGE (Log days)
N MALE
T MALE
GLUT MALE

FIGURE 3.7. Feeding rate of glut-4 males (– –) and females (——) in 12h dark:12h light (LD) photoperiod. There was no significant difference in feeding ($p>0.05$).


FEEDING RATE (Log mg dry food mass/g dry body mass/day)
3
2.9
2.8
2.7
2.6
2.5
1.5
1.75
2
2.25
2.5
2.75
AGE (Log days)
GLUT MALE
GLUT FEMALE

SECTION IV

EFFECTS OF CONSTANT LIGHT VERSUS A 12h DARK, 12h LIGHT PHOTOPERIOD ON TIME BUDGETS AND CIRCADIAN PATTERNS OF BEHAVIOUR IN GIANT MICE TRANSGENIC FOR RAT GROWTH HORMONE

4

4.1 RATIONALE AND OBJECTIVES

This thesis explores alterations in feeding, reproduction in mice expressing mulitiple copies of rat GH genes, and further impacts of a 12h:12h (LD) versus 24h light (LL) photoperiod. The main objective was to examine altered functions and structure of transgenic rat GH mice (TRrGHm) compared to normal controls, and to gain insight into altered neuroendocrine integration particularly with respect to reproductive impairments the GH mice. The study involved monitoring of i) life-history features (body size, fecundity, organ sizes), ii) feeding rates, and iii) behavioural time budgets (sleeping, locomotion, wheel running, feeding, drinking).

The following paper addresses the third component of organismal designs; specifically the impact of photoperiod on behavioural time budgets and circadian patterns to determine if behavioural energetic demands are altered in LL versus LD. This was done to evaluate whether altered behavioural costs may have contributed to accentuated infertility in LL-reared females, that is, increased activity without increased feeding would accentuate energetic shortfalls likely contributing to infertility in female TRrGHm.

4.2 CLARIFICATION OF CONTRIBUTIONS

This study was proposed by C. David Rollo, as a component of a larger research endeavor examining altered allocation and functions in TRrGHm. The current study examined the impact of constant light on reproduction, organ allometries, feeding behaviour, and activity of TRrGHm and normal controls of both sexes.

My contributions were as follows:

1. **Intellectual Contributions:**

   (i) Review of scientific literature on transgenic GH animals, impacts of constant light on feeding, activity, and sleep in rodents, and neuroendocrinological data was conducted largely by myself.

   (ii) The theories and ideas presented in this thesis were synthesized jointly by Dr. C.D. Rollo and myself.

2. **Standard Animal Care of Main Breeding Colonies:**

   All aspects of animal care were performed in conjunction with Dr. L. Kajiura.

   Status of Health

   (i) The status of health was checked for all animals daily. Cages were inspected for dead animals. Identification numbers, sex, birthdate, date of death, coat colour, strain (transgenic or normal), and diet treatment were noted for the deceased. I was specifically responsible for culling the colony, and euthanizing any ill or injured mice.

   (ii) Cages were checked daily for pregnant females and new litters. Identification numbers of parents, sex, coat colours, strain (transgenic or normal), birthdate of

offspring, and offspring body mass were recorded to complile data on the reproduction for transgenic and normal strains. At 20 days of age, mice were weaned to form new breeding groups or to be used for experimental purposes.

Food

Cage hoppers were stocked daily with standard rodent food pellets (LabDiet®, No. 5001, PMI Feed Inc., APPENDIX A) provided *ad libitum.*

Water

Water bottle were topped up daily. All bottles were washed with detergent and sterilized at three-day intervals.

Animal Breeding Cages

Cages (length x width x height = 28 x 17.5 x 5 cm), which contained 1 male and 4 females were washed, disinfected, and bedding material (wood chips) were replaced every 3 days.

3. Maintenance of Animal Quarters:

Floors, holding racks, and bench tops were washed and disinfected daily. Room temperature and photoperiod (L:D 12h:12h; L:L 24h) were also checked each day.

4. Experimental Design, Preparation, Data Documentation, and Statistical Analyses for Activity Study:

(i) Preparation and cleaning of containers for behavioural time budgets and weighing of mice before taping.

(ii) Taping of mice for 24 hours.

(iii) Collating of tapes for locomotion, exercise, feeding, drinking, and sleep.

(iv) Statistical comparisons (ANOVA) were conducted using JUMP.

4.3

Effect of constant light versus 12h dark, 12h light photoperiod on time budgets and circadian patterns of behaviour in giant mice transgenic for rat growth hormone.

by

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4.4 ABSTRACT

TRrGHm posess multiple copies of rat growth hormone genes resulting in doubled normal growth rates and adult sizes. Excessive allocation of energy into growth results in energetic trade-offs by which activity is reduced and time spent sleeping is increased (Lachmansingh and Rollo 1994; Rollo *et al.* 1997). Photoperiod entrains circadian rhythms of many behaviours including feeding, activity, and sleep. Neuroendocrines and continuous light (LL) results in the damping out and shifting of circadian rhythms. TRrGHm represent a state of hormonal imbalance likely to be impacted by photoperiod alterations. LL-reared TRrGHm on standard diet with carbohydrate and fat supplements slept less (81%, $p<0.000$) and exercised more (262%, $p<0.004$) when compared to LD-reared TRrGHm on standard diet only. A 143% increase ($p<0.03$) in drinking was also observed in LL than in LD, as well as a slight non-significant reduction in feeding. TRrGHm reared from birth in LL were more behaviourally lethargic than those reared in LD on a standard diet with carbohydrate supplements. They ate and drank slightly less and more time sleeping although these results were not significant. Circadian patterns of feeding and sleep were shifted and damped out in LL, whereas ultradian rhythms persisted. The altered rhythms observed in LL likely arose from corticosterone alterations, which correlate positively with activity and inversely with sleep.

4.5 INTRODUCTION

TRrGHm chronically express plasma rat growth hormone (rGH) at levels 100 to 400 times normal (Palmiter *et al.* 1982). The transgenes are expressed in liver, thus escaping hypothalamic-pituitary regulation. Negative regulatory feedback elecited by high levels of rGH suppress endogenous mouse GH (Bartke *et al.* 1994). Thus TRrGH mice provide a system where a crucial hormonal axis is augmented, and its temporal variation obliterated. Growth rates among lines of transgenic GH mice do not correlate strongly to plasma GH at high levels, but instead correlate to plasma levels of the downstream effector of GH, insulin-like growth factor 1 (IGF-1). This probably reflects saturation of growth hormone receptors or IGF receptors. Only minor circadian variation was detected in transgenic human GH mice considered to be functionally insignificant (Portanova *et al.* 1990).

Lachmansingh and Rollo (1994) showed that TRrGH mice were behaviourally lethargic and slept 3.4 hours/day more that normal controls. GH is strongly associated with sleep in many species (Drucker-Colin *et al.* 1975; Stern *et al.* 1975; Mendelson *et al.* 1980; Kaler *et al.* 1986; Obal *et al.* 1988; Laurentie *et al.* 1989; VanCauter *et al.* 1990, 1992a, b). They postulated that enhanced sleep resulted from an increased allocation to growth at the expense of waking (i.e. a trade-off between sleep-associated growth and behavioural activity). This was substantiated by the fact that sucrose supplements, an efficient source of metabolic energy, normalized behavioural activity and sleep of TRrGH mice (Rollo *et al.* 1997).

Many physiological functions show approximately 24h circadian rhythms entrained to external cues, particularly alterations of light and darkness. In many species, prolonged exposure to LL results in damping of circadian rhythms and arrythmicity (Aschoff 1969; Bunning 1973; Pittendrigh 1974; Honma and Hiroshige 1978; Terman and Terman 1980). Circadian rhythms in rodents also damp out in dim constant light (Albers *et al.* 1981),

constant darkness (Metler *et al.* 1977) or after blinding (Ibuka *et al.* 1977). The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the primary circadian pacemaker in mammals. Destruction of the SCN abolishes circadian rhythmicity (Moore 1978; Rusak and Zucker 1979). Light stimuli shift behavioural rhythms and regulate transcriptional events in the SCN (Kornhauser *et al.* 1992; Ginty *et al.* 1993), resulting in entrainment of the endogenous circadian clock.

There are three possible mechanisms of the arrythmicity observed in LL: 1) cessation of a single central circadian pacemaker (SCN), 2) desynchronization within a large population of oscillators that comprise the pacemaker and, 3) uncoupling of overt functions from the pacemaker (Bunning 1971; Pittendrigh and Daan 1976; Enright 1980; Wever 1980). The purpose of the present study was to observe time budgets and circadian rhythms of sleep and feeding in LL and compare these results to results previously obtained for TRrGHm in a 12h:12h (LD) photoperiod.

4.6 METHODS

4.6.1 *Animals Used*

TRrGHm were obtained from our breeding colony. This strain was engineered by Palmiter *et al.* (1982) through micoinjection of fusion genes (metallothionein-1 promoters fused to rat structural genes) into the pronuclei of fertilized mouse eggs. The eggs were subsequently implanted into foster mothers. This resulted in several copies of rGH genes being incorporated into one chromosome, which caused plasma rGH levels to rise 100-400 times normal levels (Palmiter *et al.* 1982; Shea *et al.* 1987). The inheritence of these genes is Mendelian. Hence, heterozygously transgenic fathers mated to normal females generate equal numbers of transgenic and normal animals. This provided control for genetic background.

4.6.2 *General Rearing Conditions*

Breeding groups (1 male mated to 4 females) were housed in clear plastic cages (length x width x height = 28 x 17.5 x 5 cm) at 22 +/- 2° C with a photoperiod of 24h light. A stainless steel hopper, placed over each container supported food pellets and a water bottle. All animals were fed *ad libitum* (Lab Diet®, No. 5001, PMI Feed Inc.). Cages were bedded with BetaChips® (Hardwood Laboratory Bedding) and cleaned every # days. Each cage contained an enrichment bottle or plastic tube. All protocols were consistent with the guidelines of the Canadian Council on Animal Care.

4.6.3 *Activity Containers*

TRrGH males of varying ages were preweighed and placed individually in an activity arena for 24 hours prior to the experiment to allow acclimation. The arena consisted of 4 compartments, each opening centrally into a common central "choice" compartment. The four larger compartments each contained one of the following: water bottle, running

wheel, bedding material, or food. Mice were given a free *ad libitum* choice among lard, sucrose, or the standard Lab Diet. Each food type was supplied in 5.3 cm diameter petri dishes placed equidistantly at the back of the food compartment. Dishes were fastened into place with tape. Lab Diet pellets were glued to the bottom of the dish with RTV 108 Silicone Rubber Adhesive Sealant, GE Canada Inc.®, arranged so the mice could not contact the adhesive.

4.6.4 *Variables Investigated*

After the 24 h of acclimation, each individual was video taped for a further 24 hours. Videotapes were analysed for sleeping and feeding patterns, and compared to previously recorded data taken in LD. Food choices varied from earlier studies so only total time spent feeding was evaluated for the current study.

4.6.5 *Analytical Method*

Behavioural time budgets were calculated in minutes. Overall means (+/- S.D.) were calculated for each variable. Statistical analysis consisted of analysis of variance (ANOVA).

4.7 RESULTS

4.7.1 *Impact of LL on Overall Time Budgets*

Tables 4.1 and presents the results for TRrGH males on standard diet in LD compared with those on standard diet with carbohydrate and fat supplements in LL. To facilitate comparisons the data were also calculated as percentages of the results previously recorded for LD (Rollo *et al.* 1997). LL mice slept less (81%, $p<0.000$) and were more active. Exercise increased 262% ($p<0.004$) and a non-significant increase (124%) in locomotion was observed in LL. LL-reared TRrGHm showed a 143% increase in time spent drinking ($p<0.03$) and a small non-significant reduction in feeding when compared to LD.

Table 4.2 compares TRrGH males on standard diet with carbohydrate supplements to our LL-reared TRrGH males on carbohydrate and fat supplement. None of the results obtained were significant although several trends were observed. The LL mice showed reduced locomotion and exercise. Total locomotion was only 84%, and total exercise was only 60% that of LD mice. Total time sleeping was elevated in LL by 108%. Time spent feeding or drinking was reduced to 75% and 56% that of LD mice respectively.

4.7.2 *Circadian Temporal Organization of Behaviour*

The two behaviours of greatest interest for the present study were sleep (the inverse provides waking), and feeding. Figures 4.1 and 4.2 present the mean durations of these activities for all animals for each half hour period across the 24 hour cycle. Since we filmed each animal for only 24 hours, we did nor apply statistical analysis, but the graphical presentation of averaged data provides a clear qualitative picture of circadian patterns.

Figure 4.1 shows that sleep was distributed throughout the 24 hour cycle. In LD, sleep predominated during the light phase. In LL, this pattern was reversed with most

sleep occuring during the nighttime. The circadian pattern appears slightly blunted in LL, since peak values were less than those observed in LD.

Patterns of feeding were the inverse of sleeping. In LD, most of the feeding took place in the dark (Fig. 4.2). Feeding peaked immediately after lights off, and then declined gradually throughout the dark and subsequent light period. In LL, the circadian feeding pattern was severely blunted. A slight rise in feeding occured at what would have been the beginning of the dark period, followed by a rapid decline in feeding behaviour. Feeding then gradually increased over the rest of the 24 hour period. The data indicates, however, that ultradian rhythms survived the alterations of circadian organization in LL.

4.8 DISCUSSION

TRrGHm in LD exhibit behavioural lethargy, demonstrating reduced activity and increased sleep. Increased dietary carbohydrate normalized behaviour of TRrGHm (Rollo *et al.* 1997). In LL-reared TRrGHm, carbohydrate and fat supplements also normalized behaviour (Table 4.1), to a lesser degree, which is suggestive of LL influences on behaviour.

Prolonged exposure to LL reduced durations of feeding and drinking (Table 4.2). Studies indicate that adrenergic systems in various hypothalamic sites play an important role in the control of food intake. In satiated rats norepinephrine (NE) stimulates feeding behaviour through an alpha2-adrenergic mechanism (Leibowitz 1970, 1981; Marino *et al.* 1983). In contrast, NE injections inhibit feeding behaviour in hungry rats through a beta-adrenergic mechanism (Leibowitz 1973, 1981; Leibowtiz and Rossakis 1978). Circadian rhythms in alpha2- and beta-adrenoreceptor binding have been described in various brain regions (Kofka *et al.* 1981, 1983; Wirz-Justice *et al.* 1983; Krauchi *et al.* 1984). For example, Krauchi *et al.* (1984) measured alpha2- and beta-adrenoreceptor binding throughout 24 hours in the medial and lateral hypothalamus respectively. In the lateral hypothalamus beta-adrenoreceptor binding followed a bimodal pattern with peaks at dawn and dusk. They found that when the dusk peak was eliminated food intake increased. In the medial hypothalamus, alpha2-adrenoreceptor binding showed a clear circadian rhythm with a maximum at the beginning of the dark cycle and a minimum at dawn. In LL, decreased feeding could arise if the nocturnal peak in alpha2-adrenoreceptor binding is reduced or absent, or beta-adrenergic pathways are enhanced.

CORT also affects feeding behaviour. It is normally coupled to adrenergic mechanisms which regulate feeding (Krauchi *et al.* 1984; Jhanwar-Uniyal *et al.* 1985; Bhakthavatsalam and Leibowitz 1986). Circulating CORT peaks at dark onset (Krieger

and Hauser 1978; Wilkinson *et al.* 1979). Feeding elecited by NE is attenuated or abolished by adrenalectomy and can be restored by CORT (Leibowitz *et al.* 1984; Roland *et al.* 1987), suggesting that these systems were functionally linked. In LL, circadian patterns of CORT shift and/or damp out, with ultradian patterns remaining intact (Takahashi *et al.* 1977; Honma and Hiroshige 1978) similar to feeding patterns observed in our mice in LL (Figure 4.2). Disruption of CORT (HPA axis) could impact feeding via alterations in adrenergic mechanisms. Rosenwasser *et al.* (1981) demonstrated similar feeding patterns in LL treated rats (i.e. circadian arrythmicity with intact ultradian patterns of feeding). Honma and Hiroshige (1978) also found intact ultradian rhythms of locomotion, body temperature, and corticosterone in LL-reared rats.

Several studies have examined the impact of LL on locomotor activity (Honma and Hiroshige 1978; Albers *et al.* 1981; Mason 1991; Honma *et al.* 1996). LL results in arrythmicity. LL dampens the amplitude of circadian rhythms without suppressing ultradian rhythmicity (Honma and Hiroshige 1978; Albers *et al.* 1981). The breakdown of temporal organizations is characterized by short bursts of activity (approximately 2.5 hours) (Albers *et al.* 1981). This is also consistent with the reduced activity (locomotion and exercise, Table 4.1) of LL-reared TRrGHm, and of short bursts throughout the 24 hour period. Honma and Hiroshige (1978) demonstrated that a rise in CORT precedes bursts of increased activity in LL. Since circadian patterns of CORT are blunted, and ultradian patterns are intact, it is likely that CORT contributes to activity patterns observed in LL treated TRrGHm.

Sleep may also have affected activity patterns in LL. Sleep showed a unimodal pattern with a 12h phase shift (Figure 4.1). Amplitude of sleep was less than that observed in TRrGHm in LD. Overall more time was spent sleeping (extended duration), even though amplitude was blunted. Ultradian cycles of sleep remained intact. CORT cycles vary inversely with those of sleep, and both demonstrate ultradian rhythms with similar periods

(Mitsugi and Kimura 1985). Therefore, ultradian cycles of CORT might contribute to the observed wake-sleep cycles of TRrGHm in LL. Significantly, Honma and Hiroshige (1978) showed that CORT rhythms shift by 12 hours in LL, a result that would explain the 12h shift observed in wake-sleep patterns found in our TRrGHm.

In conclusion, feeding and activity were reduced in LL, with increases in sleep. Circadian rhythms of behaviour and physiology dampen and shift in LL, possibly due to correlated changes in CORT. Ultradian patterns remained intact which would account for the short bursts of activity seen in LL-reared TRrGH mice.

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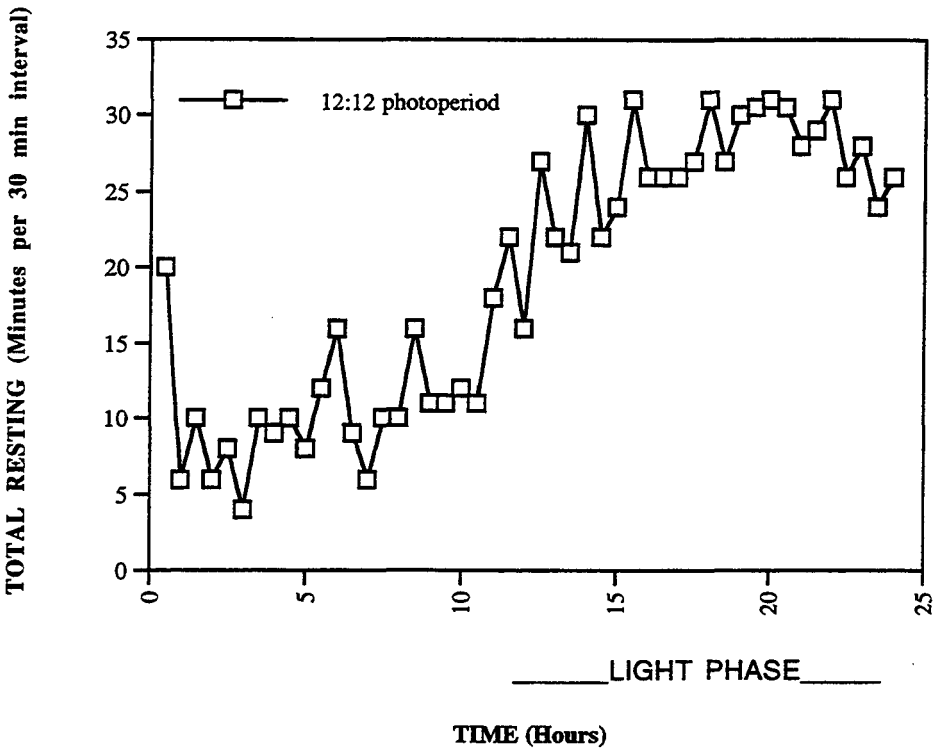
TABLE 4.1. Comparison of time budgets among TRrGHm on standard chow in 12h dark:12h light (LD) versus TRrGHm on standard chow with carbohydrate and fat supplements in 24h light (LL) photoperiods. Results are shown in minutes (other than for mass), and planned comparisons are calculated as percentages. (*) indicates significance ($p<0.05$).

VARIABLES	LL	LD	LL/LD%
Mass (g)	47.5 +/- 6.0	52.9 +/- 6.1	89.8
Locomotion	226.0 +/- 86.8	181.6 +/- 48.6	124.5
Exercise	65.8 +/- 79.9*	25.2 +/- 30.1*	261.6
Resting	144.7 +/- 60.4	112.5 +/- 52.3	128.6
Sleeping	808.8 +/- 180.7*	997.1 +/- 93.1*	81.1
Total Feeding	93.8 +/- 47.2	111.0 +/- 33.0	84.5
Drinking	14.1 +/- 6.6*	9.88 +/- 4.1*	142.7

TABLE 4.2. Comparison of time budgets among TRrGHm on standard chow with carbohydrate supplements in 12h dark:12h light (LD) versus TRrGHm on standard chow with carbohydrate and fat supplements in 24h light (LL) photoperiods. Results are shown in minutes (other than for mass), and planned comparisons are calculated as percentages.

VARIABLES	LL	LD	LL/LD%
Mass (g)	47.5 +/- 6.0	44.9 +/-8.8	105.8
Locomotion	226.0 +/- 86.8	269.8 +/- 142.2	83.8
Exercise	65.8 +/- 79.9	108.9 +/- 81.9	60.4
Resting	144.7 +/- 60.4	156.2 +/- 47.9	92.6
Sleeping	808.8 +/- 180.7	747.6 +/- 190.3	107.2
Total Feeding	93.8 +/- 47.2	125.2 +/- 37.5	74.9
Drinking	14.1 +/- 6.6	25.2 +/- 20.7	56.0

FIGURE. 4.1. Sleeping of transgenic mice in 12h dark:12h light photoperiod and 24h light over a 24 hour period. Each point represents the mean duration of sleep within each 30 minute period for all the mice in each treatment.


TOTAL RESTING (Minutes per 30 min interval)
35
30
25
20
15
10
5
0
12:12 photoperiod
0
5
10
15
20
25
LIGHT PHASE
TIME (Hours)

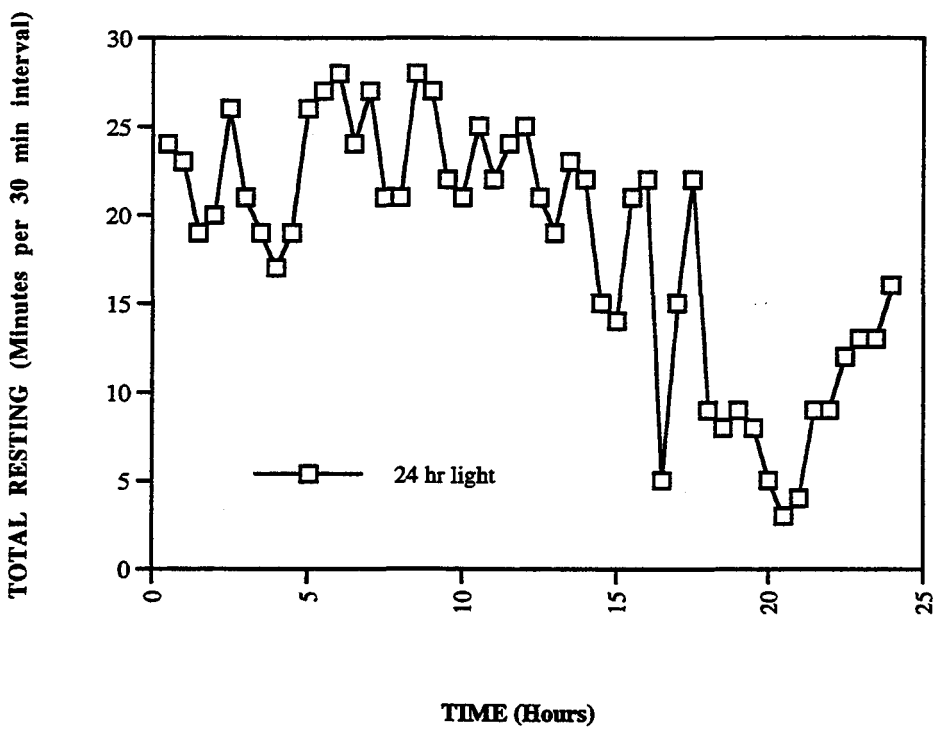
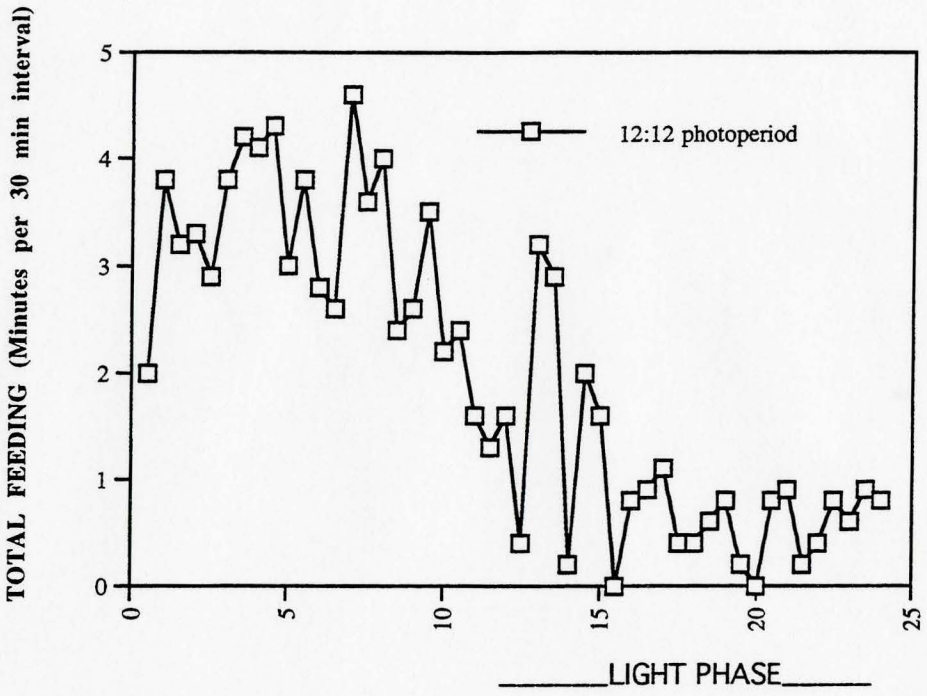
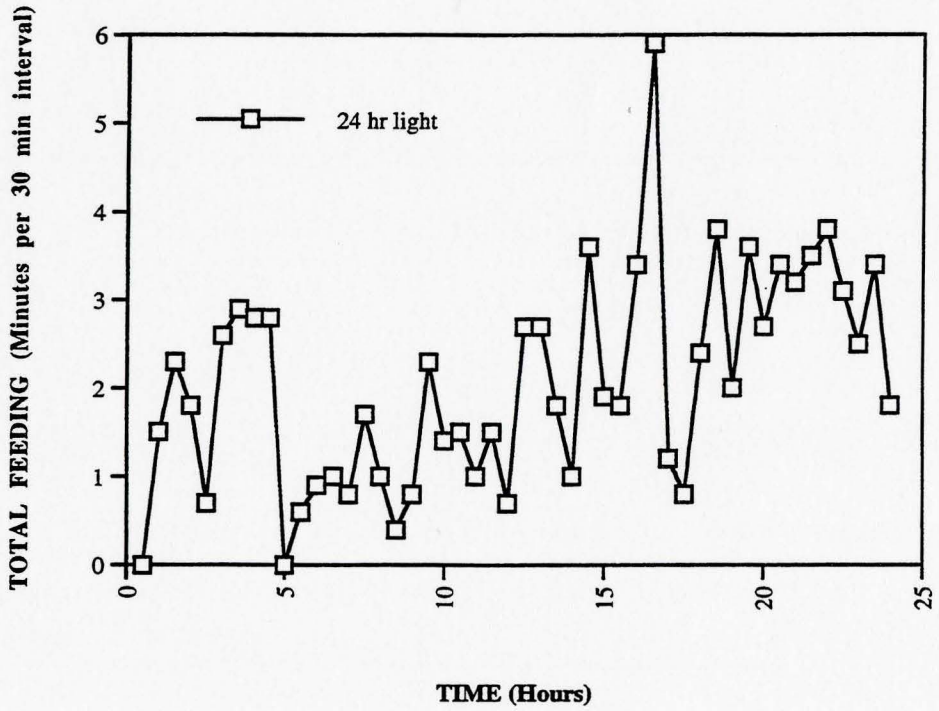

TOTAL RESTING (Minutes per 30 min interval)
30
25
20
15
10
5
0
24 hr light
0
5
10
15
20
25
TIME (Hours)

FIGURE. 4.2. Feeding of transgenic mice in 12h dark:12h light photoperiod and 24h light over a 24 hour period, when fed standard diet with a carbohydrate supplement. Each point represents the mean duration of total feeding within each 30 minute period for all the mice in each treatment.


TOTAL FEEDING (Minutes per 30 min interval)
12:12 photoperiod
LIGHT PHASE
TIME (Hours)


TOTAL FEEDING (Minutes per 30 min interval)
24 hr light
TIME (Hours)

SECTION V

CONCLUDING DISCUSSION AND GENERAL SUMMARY

5

CONCLUDING DISCUSSION AND GENERAL SUMMARY

In this thesis, the impact of photoperiod (continuous light (LL) versus 12h:12: (LD)) on various physiological processes was evaluated through comparisons of TRrGHm with their non-transgenic relatives. Of particular interest was how LL influenced reproduction, feeding, and behavioural activity. A drastic reduction (50%) in fertility (which was already low) was observed in TRrGH females reared in LL. Although normal females also demonstrated impaired reproduction (14%), this was much less than TRrGH females. Infertility may have been aggravated by reduced feeding of TRrGH females. In LL TRrGH females consumed less food (85%) than those reared under LD photoperiod. This would accentuate energetic stress, a key factor we proposed as underlying infertility in TRrGHm. TRrGHm were more behaviourally lethargic in LL, reducing activity levels and increasing sleep. Although this could compensate for (or contribute to) reduced energy intake, it was not sufficient to prevent further reductions in fertility.

Feeding and reproduction are closely linked in mammals (Piacsek and Meites 1967; Howland and Ibrahim 1973; Campbell *et al.* 1977; Sen *et al.* 1979; Dyer *et al.* 1985; McClure and Saunders 1985; Thomas *et al.* 1990; Cagampang *et al.* 1990, 1991, 1992; Wade and Schneider 1992; Helmreich and Cameron 1992; Schreihofer *et al.* 1993; Li *et al.* 1994; Nagatani *et al.* 1994, 1996a, 1996b; Murray and Pomp 1995; Bucholtz *et al.* 1996; Chen *et al.* 1996; Murahashi *et al.* 1996; Wade *et al.* 1996; Williams *et al.* 1996; Rollo *et al.* 1997). Food deprivation in female rats results in suppressed pulsatility of LH release (Dyer *et al.* 1985; McClure and Saunders 1985; Cagampang *et al.* 1990). Even though transgenic GH mice exhibit normal mean LH levels (Bartke *et al.* 1994), energetic stressors act mainly via suppression or alterations in patterns of pulsatility, and LL-reared TRrGHm may be impacted in this manner. Several factors contribute to LH suppression in fasted

rodents. In ovariectomized (OVX) rats, both alpha-adrenergic receptor antagonists (Gnodde and Schuiling 1976; Weick 1978) and agonists (Gallo and Drouva 1979; Leung *et al.* 1981) inhibit LH release. In contrast, alpha-adrenergic agonists stimulate LH release in steroid-primed OVX rats (Krieg and Sawyer 1976; Gallo and Drouva 1979; Leung *et al.* 1981). In these studies, the ovarian steroids used appeared to be important in determining whether the inhibitory or excitatory component of the adrenergic receptors dominate the mode of LH release. In fasted OVX estrogen-primed rats, alpha2-adrenergic receptor antagonists reinstated fasting-induced suppression of LH release (Cagampang *et al.* 1992). This suggested that alpha2-adrenergic receptors are specifically activated in the fasted condition in the presence of estrogen to suppress LH secretion. This implies that nutritional state is as important as the steroids in determining which alpha subtype dominates the mode of LH release.

The hyptothalamic paraventricular nucleus (PVN) is the site where alpha2-adrenergic receptors mediate the fasting-induced suppression of LH release. Food deprivation may activate a catecholaminergic projection from the nucleus of the solitary tract to the PVN (Maeda *et al.* 1994; Nagatani *et al.* 1994). Norepinephrine release in the PVN increases the negative feedback sensitivity of LH-releasing hormone (LHRH) to estrogen, perhaps by increasing estrogen receptors in the PVN (Panicker *et al.* 1995; Maeda *et al.* 1996; Nagatani *et al.* 1996). Estacio *et al.* (1996) demonstrated that the fasting-induced increase in estrogen receptors of the PVN and nucleus of the solitary tract was mediated by the vagus nerve.

Activation of estrogen-sensitive cells possibly inhibits LHRH secretion via release of corticotropin-releasing hormone (CRF) (Rivier and Vale 1984; Petragalia *et al.* 1986; Almeida *et al.* 1988; Nappi and Rivest 1995; Akema *et al.* 1996). Endogenous opioids are also involved in inhibition of LHRH release (Dyer *et al.* 1985; Petragalia *et al.* 1986; Almeida *et al.* 1988; Cagampang *et al.* 1991; Kimura *et al.* 1995; Akema *et al.* 1996). For

example naloxone, a potent opioid antagonist, restores fasting- or stress-induced suppression of LH release.

Naloxone blocks CRF mediated inhibition of lordosis in female rats, so it was suggested that CRF stimulates release of central opioids (Sirinothsinghji 1985). Subsequently, Almeida *et al.* (1988) showed that naloxone attenuated CRF-induced suppression of LH in rats. This supported previous findings by Nikolarakis *et al.* (1986) and Rivier *et al.* (1982) who demonstrated that CRF stimulated beta-endorphin release. Furthermore rats treated with antibodies against endogenous opioids showed a reduced response to the LH-suppressive effects of CRF (Almeida *et al.* 1988).

Several neuropeptides regulate both energy metabolism and reproduction, including cholecystokinin (CCK), neuropeptide Y (NPY), opioids, corticotropin-releasing factor, galanin, serotonin, and catecholamines. For example, NPY is elevated in the PVN under energy restriction (Lee *et al.* 1994), likely inhibiting both growth and reproduction (Rettori *et al.* 1990; Wade and Schneider 1992; Catzefelis *et al.* 1993; Chan *et al.* 1996; Pierroz *et al.* 1996). Normally, NPY synergizes the LH surge later in the estrous cycle, but under conditions of energetic stress, prevents it (Catzefelis *et al.* 1993). In transgenic bovine GH mice, NPY antibodies elevated LH in both intact and castrated males (Ghosh *et al.* 1991) suggesting NPY-mediated inhibition of LHRH/LH. If TRrGHm in LL have accentuated energetic stress, NPY-mediated LH suppression would be enhanced.

The gut-brain peptide CCK occurs in brain regions containing gonadal steroid receptors, and those associated with feeding and reproduction (Micevych *et al.* 1987, 1988). Hashimoto and Kimura (1986) demonstrated an inhibition of gonadotropin secretion by CCK. LHRH containing cells in the medial preoptic area receive CCK fibres and CCK impacts LH secretion from there.

Constant exposure to estrogen inhibits secretion of LHRH and consequently reduces pituitary LH release and blocks ovulation (Goodman and Knobil 1981). In addition to the

effects on reproduction, ovarian hormones modulate both growth and food intake in a variety of mammals including rats (Gray and Wade 1981; Gray and Greenwood 1982; Butera and Beikirch 1989; Sarkar *et al.* 1989; Sequet and Rowland 1990). Treatment with estradiol results in transient decreases in food intake. Since TRrGH females in LL exibited both decreased food intake, and enlarged ovaries compared to those in LD, estrogen may play a role in the reproductive inhibition observed in these mice. DeCatanzaro and MacNiven (1992) argued that estrogen primarily mediates stress-induced pregnancy blockage in mice, consistent with CRF and opioid-mediated suppression of LH release in underfed rats. Minute doses of estrogen disrupt implantation, and stress-induced infertility was alleviated by estrogen antibodies (deCatanzaro *et al.* 1994). Measurements of estrogen levels in TRrGHm in the photoperiod regimes applied here would clarify estrogen involvement in the reproductive deficiency of TRrGHm.

There are three major hormone systems implicated in sleep-wake regulation. Insulin promotes sleep (Daguir 1990) and TRrGH mice are hyperinsulinemic. In addition, GH and the hypothalamic-pituitary-adrenal (HPA) axis interact antagonistically in sleep-wake regulation. GH and GHRH secretion are strongly associated with sleep, and TRrGHm are behaviourally lethargic and sleep more (Lachmansingh and Rollo 1994). The HPA axis is conteregulatory to GH, and several studies support a role of the HPA axis in increased behavioural activity (Sutton *et al.* 1982; Ehlers *et al.* 1986; Krahn *et al.* 1988). In TRrGHm, the HPA axis is elevated (Cecim *et al.* 1992), and reproduction and feeding data suggest that it may be further increased in LL. However, elevations in the HPA axis do not overrule the effects of GH (or growth would be suppressed). Also, it is necessary to consider that LL-reared TRrGH mice exhibit increased energetic stress as a result of underfeeding, and according to the principle of allocation, physiological processes must be compromised. Therefore, in addition to impaired reproduction, reduced activity may have been a consequence or a compensatory adjustment for effective partitioning of resources.

In summary, LL negatively impacts reproduction, feeding, and behavioural activity in TRrGHm. Several neuroendocrine systems link feeding and reproduction, including the hypothalamic-pituitary-ovarian axis, the HPA axis (CRF, CORT, catecholamines, and endogenous opioids). These systems interact to regulate energy and during deficiency downregulate pathways, such as LHRH release, to limit reproductive energy expenditure. Observations suggest that both reproduction and activity are compromised as a result of the limited energy supply due to underfeeding, supporting the principle of allocation, which states that limited resources must be allocated to various physiological processes, and as one process in enhanced (growth for example), others must simultaneously be compromised. In the future, hormone assays of TRrGHm in various photoperiods may be valuable probes for understanding the organization and function of relevent neuroendocrine pathways, particularly since TRrGHm were more strongly impacted by photoperiod. This would provide a step in assessing the endocrinological disfunctions associated with enforced growth, and the possibility of restoring normal functions in GH transgenic animals, including livestock.

SECTION VI

GENERAL LITERATURE CITED

6

GENERAL LITERATURE CITED

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APPENDICES

APPENDIX A

STANDARD LABORATORY RODENT DIET™

LAB DIET® No. 5001 (PMI Feeds, Inc.)

A.1 *Guaranteed Analysis*

Crude protein not less than	23.0%
Crude fat not less than	4.5%
Crude fibre not more than	6.0%
Ash not more than	8.0%
Added minerals not more than	2.5%

A.2 *Ingredients*

(Note: ingredients are listed in terms of relative quantity in order of most abundant to least abundant)

Ground yellow corn, soybean meal, dried beet pulp, fish meal, ground oats, brewer's dried yeast, alfalfa meal, cane molasses, wheat germ meal, dried whey, meat meal, wheat middlings, animal fat preserved with BHA, salt, calcium carbonate, vitamin B-12 supplement, DL-methionine, calcium pantothenate, choline chloride, folic acid, riboflavin supplement, thiamin, niacin supplement, vitamin D-3 supplement, vitamin E supplement, calcium iodate, ferrous carbonate, copper sulfate, zinc oxide.

APPENDIX A (cont.)

LAB DIET[R] No. 5001 (PMI Feeds, Inc.)

A.3 *Chemical Composition*

Nutrients: (expressed as percent of ration except where otherwise indicated)

Protein %	23.4
Arginine %	1.38
Cystine %	0.32
Glycine %	1.20
Histidine %	0.55
Isoleucine %	1.18
Leucine %	1.70
Lysine %	1.42
Methionine %	0.43
Phenylalanine %	1.03
Tyrosine %	0.68
Threonine %	0.91
Tryptophan %	0.29
Valine %	1.21
Fat %	4.5
Cholesterol ppm	270.0
Fiber (Crude) %	5.8
Neutral Detergent Fibre %	16.0
Acid Detergent Fibre %	8.2
Total Digestible Nutrient %	76.0

APPENDIX A (cont.)

Nitrogen-Free Extract (by difference) %	49.0
Gross Energy kCal/g	4.25
Physiological Fuel Value kCal/g	3.30
Ash %	7.3
Calcium %	1.00
Phosphorus %	0.61
Potassium %	1.10
Magnesium %	0.21
Sodium %	0.40
Chlorine %	0.56
Fluorine ppm	35.0
Iron ppm	198.0
Zinc ppm	70.0
Manganese ppm	64.3
Copper ppm	18.0
Cobalt ppm	0.6
Iodine ppm	0.7
Chromium ppm	1.83
Selenium ppm	0.20
Vitamins	
Carotene ppm	4.5
Menadione (added) ppm	--
Thiamin ppm	15.0

APPENDIX A (cont.)

Riboflavin ppm	8.0
Niacin ppm	95.0
Panthothenic Acid ppm	24.0
Choline ppm x 100	22.5
Folic Acid ppm	5.9
Pyridoxine ppm	6.0
Biotin ppm	0.07
B_{12} mcg/kg	22.0
Vitamin A IU/g	15.0
Vitamin D IU/g	4.5
Vitamin E IU/g	40.0
Ascorbic Acid mg/g	--

APPENDIX B

CONVERSION GRAPH

Conversion of wet mass to dry mass as a function of age.