RESOURCE ALLOCATION IN GIANT "SUPERMICE" GENETICALLY ENGINEERED WITH EXTRA RAT GROWTH HORMONE GENES

By

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ABSTRACT

Genetically engineered "Supermice" harbouring multiple copies of rat growth hormone genes, attain adult body sizes almost twice that of normal mice. To determine how transgenic mice adjust resource acquisition and processing with elevated growth, dry mass budgets were conducted on Supermice (strain Tg[MT-1, rGH], Bri2) and normal *Mus musculus*. Rates of growth, consumption, faecal deposition, digestive assimilation, respiration, and production efficiencies were compared for both early and late growth intervals.

Younger, faster-growing mice (25-40 days old) displayed higher rates and production efficiencies than those documented for older, slower-growing mice (47-62 days old). Surprisingly, Supermice never exhibited growth rates greater than those displayed by the most rapidly growing normal controls. For transgenic animals, larger body sizes were achieved by maintaining increased growth rates into later ages. Dry mass budgets revealed that Supermice failed to alter mass-specific feeding rates to compensate for their increased growth demands, but production efficiencies were greatly enhanced instead. Superior conversion of assimilated food into biomass was obtained by diverting resources from other behavioural, reproductive, and longevity assurance systems. Shortcomings prevalent in Supermice (lethargy, reduced fecundity, decreased longevity, disturbed metabolism, and various pathological problems) have been similarly expressed in genetically engineered livestock and other organisms conforming to acromegalic states including humans and large breeds of dogs. Thus, transgenic GH mice may offer practical insights for agricultural and medical applications.

Trade-offs in the Supermouse are apparent in their "transgenic correlation structure" which represents a new alternative for testing and clarifying facets of life-history theory. It is concluded that production efficiency is a key component linking life-history features in *Mus musculus*, and may be a fundamental element in both environmental adjustments and evolutionary changes.

ACKNOWLEDGEMENTS

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"The Big E" is gratefully acknowledged for his kindness during my post-ACL knee surgery rehabilitation. Special thanks go to "tenacious" Trev, who has inspired me to embrace all challenges with "a 110% effort". Extra special thanks go to Jacques for all the FUN times.

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SECTION III

PREFACE

The primary goal of our Supermouse research programme was to extend knowledge concerning the physiological, behavioural, and ecological implications of genetically engineered growth enhancement in vertebrates. The emphasis of the present study centres on the altered physiological resource allocation and associated trade-offs of giant "Supermice", 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 field of evolutionary ecology and organism design. The "principle of allocation", a fundamental tenet of life-history theory is highlighted. Descriptions of gene transfer techniques and the specific characteristics yielded by transgenic manipulations provide essential background regarding the incorporation and expression of foreign gene constructs.

Work described in this thesis is presented in the form of two journal articles. Each manuscript is preceded by a brief description of the employed experimental approach, as well as details of my specific contributions. For material reprinted from a previously published source, a formal acknowledgement of that source is found immediately before the reproduced article. Both manuscripts have been reformatted to comply with the specifications required by the Department of Graduate Studies at McMaster University.

The first article entitled, "*A mass budget for transgenic "Supermice" engineered with extra rat growth hormone genes: evidence for energetic limitation*", was published in the

Canadian Journal of Zoology (Kajiura, L. J., and Rollo, C. D. 1994. Can. J Zool. 72: 1010-1017). This investigation explored the physiological resource allocation strategy of giant "Supermice" during their late moderate stage of growth (mice aged 50-61 days old). To assess dry mass budget differences in normal and transgenic male mice, we determined whether the two strains operated along the same resource base. Do normal and transgenic mice consume identical quantities of food with similar assimilation efficiency? Results suggested that despite having elevated growth, transgenic animals failed to alter mass-specific food consumption. Without higher levels of resource intake, the impact of enhanced growth imposed unavoidable trade-offs between integrated fitness features, congruent with the principle of allocation framework.

The second paper entitled, "*The ontogeny of resource allocation in giant transgenic rat growth hormone mice*" was prepared for publication in the Canadian Journal of Zoology. In this report, we predicted that dry mass budget rates and production efficiencies would be markedly greater during early rapid growth (mice aged 25 to 40 days old) than those exhibited during late growth periods (mice aged 47 to 62 days old) for both Supermice and normal controls. Of specific interest was the identification of resource allocation differences expressed in normal and transgenic strains at early ages. Reduced lifespan, decreased reproduction, behavioural deficits, and pathological changes inherent of transgenic mice, emerge as a consequence of altered physiological resource allocation.

Finally, the *Concluding Discussion and General Summary* draws together the overall implications of the two studies and suggests areas which demand further research.

SECTION I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 *The Holistic Realm of Evolutionary Ecology: The Principle of Allocation*

Over the last thirty years, evolutionary ecology has rapidly emerged as a recognized field of inquiry (Pianka 1978). This new discipline strives to understand the holistic integration of organismal form and function in its evaluation of adaptive suites (Stearns 1992, Rollo 1994). By focusing on whole-organism design and organismal selection, evolutionary ecology differs from strongly reductionistic perspectives prevailing in most biological sciences. Although the reductionistic scientific method is still predominant in evolutionary ecology, the theoretical perspective is necessarily more holistic. Proponents of evolutionary ecology recognize that organisms are essentially complex resource allocation systems. Within this framework, available resources are distributed amongst competing physiological demands such as growth, reproduction, defense, behaviour, maintenance, and storage. Since resource supply is ultimately constrained or limited, any increase in allocation towards a specific energetic demand will eventually compromise resources available for other requisites.

This universal paradigm is the well-founded "principle of allocation", a fundamental assumption pervading life-history theory (Sibly and Calow 1986; Rollo 1994). From the vantage of this principle, the array of functions and processes that an organism must fulfil can be envisioned as slices of a pie (Figure 1.1). Any amplification of a particular feature (or slice

of the pie) is only obtained at the expense of robbing resources from other features, hence unavoidably impinging on the size of the other pie portions. If mass-specific feeding rates (i.e. the size of the pie) remain unchanged, an exaggeration in any one component, such as growth, must impact negatively on other fitness elements. The mode in which resources are partitioned is not random but is a controlled process deemed the resource allocation strategy. Mediated by evolved endocrine signals, such as the growth hormone axis (Thissen *et al.* 1994), resource allocation patterns ultimately reflect negatively correlated relationships or "trade-offs" between various life-history characteristics and competing processes (Reznick 1985). With trade-offs strongly influencing organismal ontogeny, physiology, and behaviour, natural organisms must partition their "pies" to maximize reproduction and survival. Although trade-offs would not be necessary if acquisition and processing rates were elevated to alleviate costly resource expenditures (Tuomi *et al.* 1983), in reality, there are strong constraints on increased feeding and digestion rates.

The success of the principle of allocation stems from the fact that organismal design is sufficiently constrained (e.g. size-specific resource intakes) so that a general predictive theory seems possible. Life-history theory then emerges from the consequence that essential fitness demands must ultimately be traded-off against one another. Traditional approaches employed to detect trade-offs include: (*i*) genetic correlations in breeding populations in single environments; (*ii*) genetic correlations derived from artificial selection regimens; (*iii*) phenotypic correlations among and within experimental treatments for species or populations; and (*iv*) phenotypic correlations across and within different phylogenetic levels (Lessells 1991; Stearns 1992; Roff 1993; Rollo 1994). Until recently, only comparisons across genotypes or

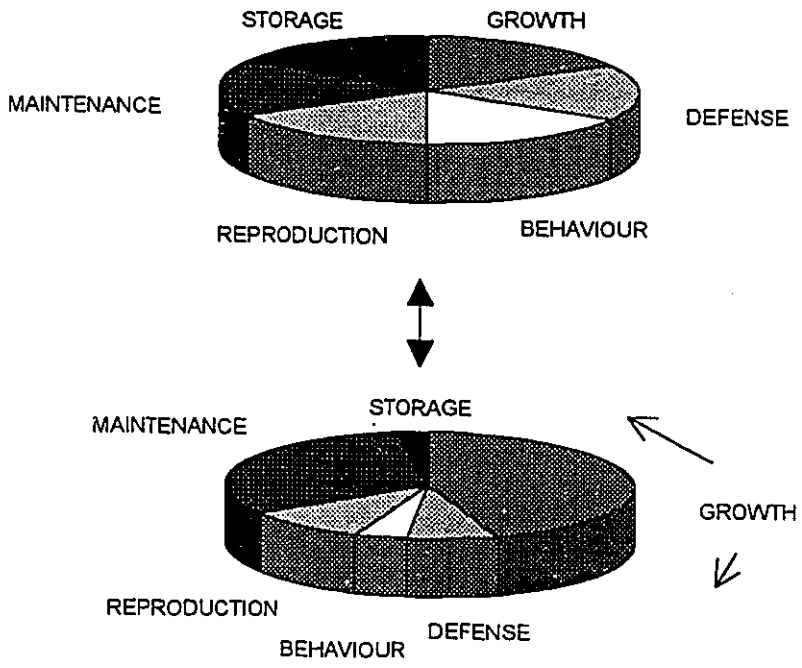

FIGURE 1.1 A conceptual model illustrating the physiological resource allocation strategy of organisms. Based on Rollo's "sliced pie "analogy, any exaggeration of a specific fitness feature (or slice) may only be obtained by diverting resources from other components, unavoidably affecting the sizes of the other pie portions.

experimental treatments under artificial selection have consistently obtained the predicted negative genetic and phenotypic correlations.

When attempting to address the principle of allocation, several confounding factors must be considered that may conceal underlying trade-offs. For example, short-term investigations may fail to resolve trade-offs if organisms efficiently utilize storage reserves. Similarly, if resource availability varies across treatments, individuals may adjust their processing rates, hence alleviating expected trade-offs. Furthermore, organisms may vary in quality due to maternal influences, age, genetic variation, inbreeding, phenotypic plasticity, disease, or mutations. Finally, detection of trade-offs may be hampered if two components, such as growth and reproduction are both enhanced, while another feature, such as longevity is simultaneously compromised. Clearly, a holistic ecological perspective is necessary to scrutinize trade-offs in complex highly-integrated organisms (Pease and Bull 1988; Rollo 1994). To successfully reap the rewards of genetic engineering, researchers must be able to predict both the positive and negative consequences (i.e. trade-offs) of genomic alterations affecting organism design.

1.2 Genetic Engineering and The Need for Ecological Involvement

Genetic engineering has been predominantly the domain of molecular biologists (Babinet *et al.* 1989; Grosveld and Kollias 1992; Murray 1992; Maclean 1994; Pinkert 1994), while involvement of ecologists remains rare. Labour and costs associated with genetically altering the genome are substantial (Gordon 1983, 1993; Brem and Muller 1994; Seamark 1994; Wight and Wagner 1994). Thus, insight into the general principles regulating organism

design may help identify which engineered features could afford the achievement of desired goals in genetically designed animal models. To date, the most difficult challenge encountered by evolutionary ecologists interested in organismal design has involved experimentally amplifying specific features to ascertain their adaptive merits. Previous efforts have been fraught with complications as selection for a single exaggerated attribute influences the "whole" organism, often with maladaptive consequences. Unknown and possibly multiple genetic alterations are the likely result. The problem is somewhat an exaggerated version of the phenomenon of "genetic slippage" seen in artificial selection programmes (Dickerson 1955), except then the problem is with genetic correlations rather than transgenic ones. Transgenic animals represent ideal genetically characterized systems with a single exaggerated or repressed genetic alteration.

1.3 Incorporation of Novel Genes into the Genome: Methods of Gene Transfer

Gordon *et al.* (1980) originated the term "transgenic" to describe animals that contained multiple copies of foreign genes (transgenes), artificially integrated into their genome. Foreign DNA may be stably introduced into the germ line using one of the following methodologies: (*i*) genetic manipulation of embryonic stem cells; (*ii*) retroviral integration into early-developing embryos; and (*iii*) pronuclear microinjection (Babinet *et al.* 1989; Boyd and Samid 1993; Gordon 1993; Evans *et al.* 1994). The latter is the current method of choice and only proven procedure for integrating foreign gene constructs into livestock.

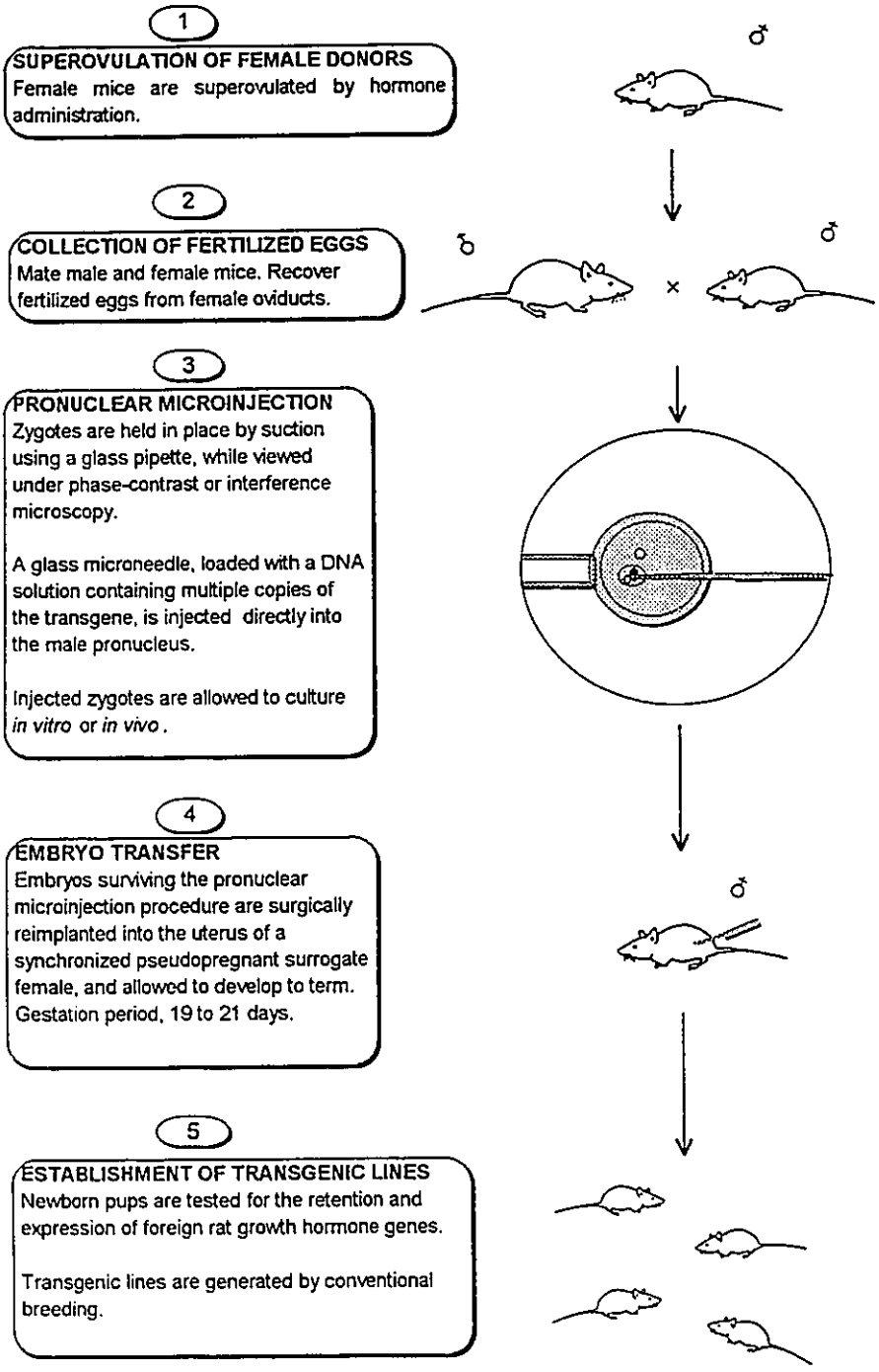

FIGURE 1.2 PRODUCTION OF TRANSGENIC "SUPERMICE" BY PRONUCLEAR MICROINJECTION

1.4 Production of Giant Transgenic-GH "Supermice"

The transgenic "Supermouse" represents an integrated genetic system with only one exaggerated alteration, that being multiple copies of rat growth hormone genes (rGH) incorporated into a single chromosome (Palmiter *et al.* 1982, 1983). Genetically engineered rodents display elevated rates of growth and achieve adult body sizes almost twice that of their normal littermates. To produce transgenic Supermice, foreign growth hormone genes are incorporated into the genome of normal mice via direct microinjection of fusion genes (metallothionein-I (MT-I)) promoters coupled to rat (rGH) growth hormone structural regions) into the male pronuclei of newly fertilized (one-cell) mouse eggs (Brinster and Palmiter 1986). Implantation of microinjected embryos into pseudopregnant surrogate females results in the generation of giant transgenic animals. Since rGH transgenes are stably integrated into a single chromosome, they are inherited as Mendelian traits. One advantage of microinjection is that large gene constructs may be introduced into the genome. A disadvantage of the method is that the identity of the specific chromosome into which the foreign gene constructs are randomly integrated remains unknown (Palmiter and Brinster 1986). Figure 1.2 summarizes the basic steps utilized to establish transgenic lines of Supermice. Detailed descriptions of gene transfer procedures are reviewed in Gordon (1993).

1.5 The Growth Hormone Cascade

Stimulatory "growth hormone releasing factor" (also referred to as growth hormone releasing hormone) and inhibitory somatostatin are hypothalamic neuropeptides, which act on the anterior pituitary to regulate the secretion of GH (Schindler *et al.* 1972; D'Costa *et al.*

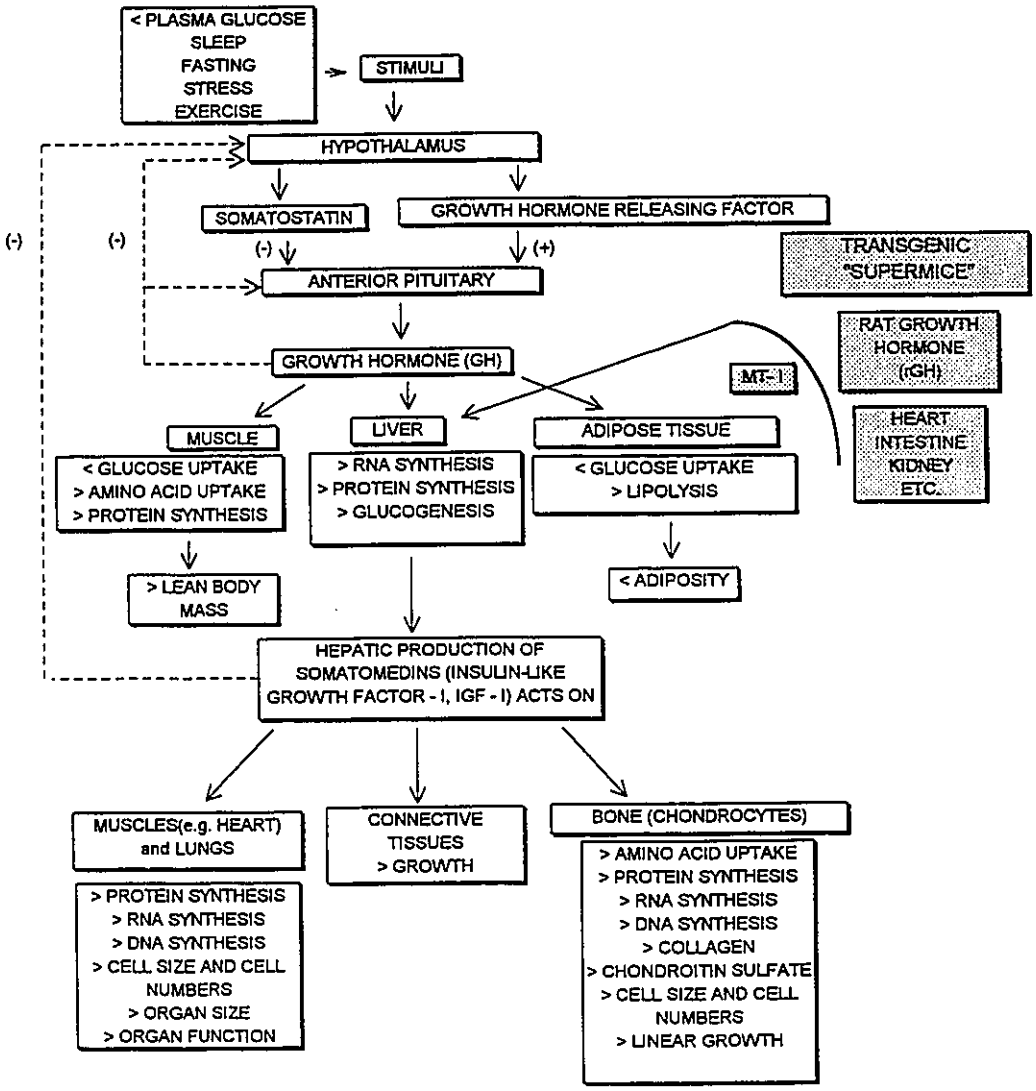

FIGURE 1.3 GROWTH HORMONE CASCADE FOR NORMAL AND TRANSGENIC MICE

1993; Spath-Schwalbe *et al.* 1995). GH induces the hepatic production of somatomedins such as insulin-like growth factors I (IGF-I) and II (IGF-II), which stimulate growth in various target tissues. Regulation of the endocrine cascade occurs via feedback mechanisms involving several levels of: (*i*) growth hormone, its receptor; (*ii*) insulin-like growth factors; (*iii*) their cell-surface receptors; and (*iv*) a number of binding proteins that carry these hormones in serum and influence their interactions with receptors (Froesch *et al.* 1985; Thissen *et al.* 1994; Wolf *et al.* 1994; Zarrilli *et al.* 1994). Mice expressing extra rGH genes possess serum GH levels 100 to 400 times those of normal mice (Brem *et al.* 1989). As shown in Figure 1.3, the MT-I promoter is primarily activated in the liver. Foreign growth hormone genes circumvent normal physiological regulatory mechanisms resulting in life-long excesses of synthesized rat growth hormone. At the same time, production of endogenous GH by the pituitary is inhibited (Wanke *et al.* 1992; Bartke *et al.* 1994). Transgenic mice allow researchers to explore the impact of chronically elevated levels of functionally saturated GH while avoiding immunological suppression that sometimes accompanies injections of foreign GH.

1.6 Induction of Transgene Expression: The Use of Promoters

Under the control of endogenous regulatory sequences, human (h), and rat (r) GH transgenes failed to enhance growth in mice (Wagner *et al.* 1983; Hammer *et al.* 1984). This suggested that foreign GH genes, which were ligated to endogenous promoters, were constrained by endogenous regulatory mechanisms. Integrated gene constructs consisting of rGH genes fused to mouse metallothionein-I (MT-I promoters) regulatory sequences successfully induced elevated transcription of the foreign rGH transgenes (Palmiter *et al.*

1983; Wall *et al.* 1990). Studies utilizing metallothionein regulatory sequences fused to human (h) or bovine (b) GH structural genes have obtained congruent results in mice (Pomp *et al.* 1995). Regulatory sequences associated with the genes for albumin (alb) (Pinkert *et al.* 1987), Moloney murine leukemia virus (MMLV) (Ebert *et al.* 1988), phosphoenolpyruvate carboxykinase (PEPCK) (McGrane *et al.* 1988; Bartke *et al.* 1994), and prolactin (PRL) (First and Haseltine 1991), have been successfully employed to drive foreign gene expression in transgenic strains.

1.7 Desired Goals and Specific Applications of the Transgenic-GH System

Traditional breeding programmes are both slow and relatively imprecise (Ward and Nancarrow 1991). With increased global demands for food, attempts to enhance livestock production have focused interest in genetic farming. From an agricultural perspective, transgenic technology offers a means whereby not only novel, heritable, and economically important attributes may be introduced into a species' genome, but also existing characteristics may be modified more rapidly than by traditional selective breeding practices (Jaenisch 1988; Knapp *et al.* 1994; Muller and Brem 1994; Powell *et al.* 1994). Whereas conventional selective breeding requires several generations, significant progress can be made in one generation using transgenic biotechnology. Furthermore, gene transfer techniques enable desired attributes to be emphasized in ways which normally would not occur through natural selection or selective breeding practices due to biological barriers, such as interspecific sterility. In essense, transgenic technology offers a rapid and specifically goal-oriented avenue for achieving genomic change.

Time and cost constraints make research using large domestic animals logistically less attractive. Relevant insights into the applications necessary to improve livestock production may be obtained using a more practical mammalian model such as the house mouse, *Mus musculus*. Assets favouring the use of *Mus musculus* include: short generation periods providing large numbers of animals with specific genetic and phenotypic characteristics to be readily available (Cook 1965; Green 1966; Poiley 1972; Berny and Bronson 1992), relatively cheaper maintenance costs making studies more economically feasible (Gordon 1993), and extensive background knowledge of the species' genetics (Crispens 1975). The murine system has been successfully implemented to assess the effects of artificially selected growth on various design features including behaviour, body composition, food consumption, and overall growth efficiency (Fowler 1962; Lang and Legates 1969; Timon *et al.* 1970; Bradford 1971; Sutherland *et al.* 1974; Brown and Fraham 1975; Canolty and Koong 1976; Kownacki *et al.* 1977; Allen and McCarthy 1980; McCarthy 1980; McPhee *et al.* 1980; Gunsett *et al.* 1981; Yuksel *et al.* 1981; Malik 1984; Bishop and Hill 1985; Bernier *et al.* 1987; Bailey *et al.* 1988; Campbell *et al.* 1988; Moride and Hayes 1993). Livestock breeders are interested in applying gene transfer to improve feed efficiency, increase growth, improve animal products (i.e. lean-muscle, wool, or milk production, see Ward and Nancarrow 1991; Byers *et al.* 1993; Powell *et al.* 1994; Seamark 1994), enhance reproductive performance, or elevate disease resistance (Wall *et al.* 1992; Petters 1994). Pinkert *et al.* (1987) noted that a mere 10% increase in feed utilization efficiency and growth rate would return a one billion dollar savings for U.S. swine breeders.

Enhanced growth is one of the most coveted traits for agriculture. Breeders of large

FIGURE 1.4 The giant trangenic Supermouse and its normal littermate. *At the bottom*, is a typical genetically engineered adult male Supermouse possessing foreign gene constructs composed of metallothionein-I promoters fused to rGH structural genes. *At the top*, is a typical normal adult male *Mus musculus*.

domestic animals have previously elevated growth by artificial selection, or by treatment of growth-promoting substances (Fronk *et al.* 1983; Peel *et al.* 1983). Exogenous administration of GH in pigs (Chung *et al.* 1985) and dairy cattle (Fronk *et al.* 1983) has produced increased protein deposition, more efficient food utilization, reduced fat and increased milk production. The tedious task of injecting animals with exogenous GH could be easily eliminated if gene transfer techniques were used to produce functional and stable changes in the host mammalian genome. Since the creation of the Supermouse (Figure 1.4), diverse fields have embarked on transgenically incorporating growth-promoting genes into species including economically important livestock. So rapid is the pace, that an exclusive computerized database for transgenic animals, TBASE, has been erected on the Internet (Woychik *et al.* 1993).

1.8 The Transgenic Supermouse: A Powerful New Tool for Evolutionary Ecology

The Supermouse is highly relevant to understanding organism design since numerous life-history features scale strongly and in an integrated manner with body size (Peters 1983; Calder 1984; Schmidt-Nielsen 1984; Reiss 1989; Millar and Hickling 1991; Charnov 1993). Simple interspecific allometric equations can predict correlated changes in a variety of phenotypic attributes across a diverse range of body size. This suggests that alterations in body size may be a vehicle for integrated evolutionary change by selection on the activity or number of growth hormone genes. Does genetically engineered growth enhancement result in a "super" mouse, or rather a physiologically stressed "supermess"? Will the incorporation of multiple copies of rGH genes lead to adaptive "rat-like" features in giant Supermice or result in extreme maladaption? If the latter prevails, signficant genetic reorganization would

seem imperative to establish a coadapted balance at abnormally larger body sizes.

The Supermouse system fulfils the essential requirement for detecting trade-offs since exaggerated growth costs are not offset by increased resource intake (Kajiura and Rollo 1994). Engineered growth enhancement without greater rates of consumption, negatively impacts on other transgenic correlation characters. The Supermouse system provides evolutionary ecologists with a powerful model for testing the verity of the principle of allocation, thus offering a new dimension in perturbation analyses. An assessment of physiological resource allocation in Supermice may explain and clarify the observed functional deficits (decreased longevity, reduced fecundity, diminished behavioural activity, and pathological symptoms) endured by other transgenic strains (Wagner *et al.* 1983; Hanahan 1989; Quaife *et al.* 1989; Wanke *et al.* 1992; Steger *et al.* 1993; Bartke *et al.* 1994; Ferraro *et al.* 1994, Lachmansingh and Rollo 1994; Pomp *et al.* 1995).

It is noteworthy that neither artificial selection nor transgenic manipulations for individuals that grow faster with greater efficiency ensures the production of a "superior" strain. Because the genome is a coevolved and highly integrated entity, genetic alterations, such as those incurred by transgenesis or artificial selection necessarily impact on other organismal design features. To our knowledge, few if any, research efforts have addressed the ecological ramifications of transgenesis in mammalian models. Current understanding of physiological mass budgets, behavioural time budgets, and other life-history trade-offs for transgenic *Mus musculus* strains is deficient. In the pages to follow, I endeavour to provide an ecological assessment of the impact of genetically engineered growth enhancement on organism design. Specifically, the intent of this investigation was to assess the changes in

physiological resource allocation of transgenic mice expressing foreign rGH gene constructs. This study will hopefully underscore the importance of utilizing the comprehensive scope of ecology to successfully exploit genetically engineered target species. Although experimental verification has proven elusive, trade-offs for experimentally manipulated phenotypes or artificial selection of key fitness characters, have consistently concurred with the principle of allocation paradigm. Results reported in this thesis offer empirical support for the reality of the principle of allocation, and reveal the integration of several organism design components (life-history, bioenergetics, and behaviour). Findings confirm that transgenic Supermice are invaluable research tools, which may be employed as model systems in diverse areas of study. Specifically, we promote their use in the field of evolutionary ecology.

SECTION II

A MASS BUDGET FOR TRANSGENIC "SUPERMICE" ENGINEERED WITH EXTRA RAT GROWTH HORMONE GENES: EVIDENCE FOR ENERGETIC LIMITATION

2

A mass budget for transgenic "Supermice" engineered with extra rat growth hormone genes: evidence for energetic limitation.

(Kajiura, L. J., and Rollo, C. D. 1994. Can. J. Zool. 72: 1010-1017)

2.1 RATIONALE AND OBJECTIVES for Kajiura and Rollo (1994)

This manuscript describes the physiological resource allocation strategy of transgenic mice expressing multiple copies of rat growth hormone genes. The main objective of this project was to holistically examine the design of the genetically engineered "Supermouse" by comparing it to normal mice. Our laboratory investigated three dimensions of organism design to assess the impact of genetically engineered growth enhancement. This required detailed monitoring of: (*i*) life-history features (body size, longevity, litter sizes, lifetime fecundity, reproductive schedules, size of pups, growth rates); (*ii*) behavioural time budgets (resting, locomotion, sleeping, wheel running, feeding, drinking, grooming); and (*iii*) dry mass budgets (growth, assimilation efficiency, food consumption, production efficiency, respiration).

The present study focused on the third component of organism design, specifically how transgenic Supermice alter acquisition and processing of resources under the engineered demands of growth. Without an examination of the strain's internal resource base, it was impossible to ascertain whether trade-offs result from an internal shift in resource allocation or by alterations in food consumption and assimilation. In order to evaluate life-history trade-offs, it was crucial to obtain information concerning the physiological basis of resource allocation. The incorporation of multiple copies of rat growth hormone genes may result in

the transgenic animal expressing "rat-like" characteristics resembling interspecific shifts in allocation, or displaying elevated rates of processing to pay for the extra costs of increased growth. By comparing the physiological resource allocation strategy of transgenic rGH Supermice with that of its normal relatives, the integration of the "transgenic correlation structure" of Supermice is revealed. With this information, we assessed whether the genetically engineered manipulation represented an avenue to produce larger, more metabolically powerful species, or merely a metabolically more intense organism.

2.2 CLARIFICATION OF CONTRIBUTIONS for Kajiura and Rollo (1994)

The original idea for this study was proposed by C. David Rollo, as a component of a larger research endeavour holistically examining the behavioural time budget, physiological resource allocation, and life-history tactics of transgenic Supermice. Much of the subsequent theoretical development emerged as a collaborative synergism.

The present study provides a detailed assessment of the dry mass budget of normal mice and genetically engineered Supermice, transgenic for extra copies of rat growth hormone genes. Individual mass budgets were recorded over 247 consecutive days. During the course of this project, I also aided in the collection of life-history data (longevity, growth, and reproduction) for both transgenic and normal mice.

My contributions were as follows:

1. **Intellectual Contributions**:

   (*i*) Review of the scientific literature on transgenic GH animals, specifically on physiological resource allocation, was conducted largely by myself.

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

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

   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. Collectively, this information provided longevity data for both transgenic and normal strains. Injured animals or mice displaying unusual health symptoms were isolated from the main breeding colony pending further medical diagnosis.

(*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, diet treatment, number of offspring, and offspring body mass were recorded to compile 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 other experiments.

(*iii*) All animals maintained in the breeding colony were weighed weekly to accumulate growth data for both transgenic and normal animals.

Food:

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

Water:

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

Animal Breeding Cages:

Cages, which housed 1 male and 2 females, were washed, disinfected, and bedding material replaced every 5 days. Cages ,which held five adult mice, were cleaned at 2 day intervals.

3. **Maintenance of Animal Quarters**:

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

4. **Experimental Design, Preparation, Data Documentation, and Statistical Analyses for Mass Budget Study**:

(*i*) Preparation, construction, and assembly of general rearing containers and experimental mass budget containers (see APPENDIX B).

(*ii*) Preparation, assembly, and weighing of food pellet dishes for daily measurements of food consumption. Construction and weighing of faecal collection vessels for daily deposition measures.

(*iii*) Documentation and calculation of food consumption, faecal deposition, growth, assimilation, assimilation efficiency, respiration, gross production efficiency,and net production efficiency. Statistical comparisons (*t*-tests, correlation-regression analyses) were conducted using Excel, Versions 4 and 5 (Microsoft® Inc.), in consultation with C. D. Rollo.

2.3 *Letter of consent, from the National Research Council of Canada, granting permission to include the published journal article (Kajiura and Rollo 1994) as part of the present thesis.*

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2.4

A mass budget for transgenic "Supermice" engineered with extra rat growth hormone genes: evidence for energetic limitation.

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2.5 ABSTRACT

Genetically engineered "Supermice" (*Mus musculus*, transgenic strain Tg[MT-1, rGH], Bri2) possess multiple copies of rat growth hormone genes yielding growth rates 220% that of normal mice. To discover how Supermice alter their acquisition and allocation of resources under elevated costs of growth, a resource allocation study was conducted on forty 50-day-old normal and transgenic male mice. Individual dry mass budgets were used to compare rates of growth, consumption, faecal deposition, digestive assimilation, and respiration over 11-day intervals. The mean body mass of transgenic mice was 153% that of normal animals during this period. Surprisingly, on a mass-specific basis, Supermice consumed 6% less food despite their higher investments in growth (normal: 0.50 ± 0.01 mg food / mg dry body mass per day; Supermice: 0.47 ± 0.01 mg food / mg dry body mass per day). Assimilation efficiency was also slightly lower in Supermice (64.1%) than in normal animals (66.7%). Enhanced growth was achieved entirely through improved conversion efficiencies. Gross and net production efficiencies of Supermice were 227% and 244% those of controls, respectively. Such increased efficiencies appeared to be the result of diverting resources from processes such as behaviour, longevity assurance, and other respiratory demands. Evidence for such trade-offs supports the "principle of allocation", a key assumption for theories of life-history evolution.

2.6 INTRODUCTION

Genetic engineering allows specific modification of naturally evolved genomes. Such precise manipulations are ideal probes for understanding the integration and coordination of features and processes constituting a species' adaptive suite. Because transgenesis amplifies one specific feature and disrupts its coadaptation with others, probing the ecological function and fitness of transgenic strains provides insight into naturally evolved coadaptation, and is also essential for directing further genetic or environmental alterations to achieve desired performance.

Evolutionary ecology explores the holistic integration of organismal form and function (Stearns 1992; Roff 1993; Rollo 1994). Organisms may be considered physiological resource allocation systems, with strategic deployment of resources among conflicting demands of growth, reproduction, maintenance, defense, storage, and behaviour. Because animals have limited ability to process and acquire resources, negative trade-offs are expected among competing physiological expenditures and key fitness attributes. Enhancement of one dimension must eventually impact negatively on others, even if short-term increases in resource intake or utilization of reserves offset short-term impacts (Tuomi *et al.* 1983). Such trade-offs are the basis of the "principle of allocation" that underlies much of life-history theory (Rollo 1994).

Despite widespread acceptance of such theory (Sibly and Calow 1986; Lessells 1991; Reznick 1992; Stearns 1992; Roff 1993; Rollo 1994), affirmation has proven challenging. Lachmansingh and Rollo (1994) review the traditional approaches for addressing trade-offs.

A major difficulty has been that exaggeration of one feature oftens leads to general maladaptation. In naturally evolved animals, experimental manipulation or selection act on the entire suite of fitness features simultaneously (i.e., whole organisms), so it is difficult to target specific aspects. Genetic engineering circumvents this limitation.

The "Supermouse" provides an ideal model for testing the principle of allocation. Supermice exhibit growth rates and adult body sizes twice those of normal mice and represent a genetic system with only one alteration, multiple copies of rat growth hormone (rGH) genes incorporated into a single chromosome (Palmiter *et al.* 1982, 1983). Supermice did not increase specific feeding rates to pay the costs of accelerated growth, so negative trade-offs with other requirements are predicted.

Our purpose was to evaluate the physiological and ecological impacts of enhanced growth by examining how Supermice reallocate their resources under the demands of elevated growth. This study is one component of a larger effort that holistically assesses the impact of transgenesis on physiological resource allocation, behavioural time budgets, and life history features of mice. Lachmansingh and Rollo (1994) report the behavioural aspects. By examining the "transgenic correlation structure" expressed by Supermice, we hope to gain insight into the naturally evolved coadaptive design of the mouse.

Several agricultural species have also been engineered with extra GH genes with the goals of increased growth, improved body composition, and enhanced feed efficiencies (Hammer *et al.* 1985a,b; Campbell *et al.* 1988; Pursel *et al.* 1989, 1990; Ward and Nancarrow 1991). Attempts to "improve" livestock have been plagued by the same syndrome expressed in Supermice (lethargy, decreased longevity, and infertility). A practical insight

resulting from the research reported here is that transgenic varieties may fail to adjust food intake to compensate for their elevated costs of growth.

2.7 METHODS

2.7.1 *Animals Used*

Supermice, (*Mus musculus*, transgenic strain Tg[MT-1, rGH], Bri2), were used to establish a large breeding colony. Palmiter *et al.* (1982) engineered this strain by microinjection of fusion genes (metallothionein-1 promoters fused to rat GH structural genes) into the pronuclei of fertilized mouse eggs. These were then implanted into pseudopregnant foster mothers. Supermice have multiple copies of rGH genes incorporated into one chromosome, resulting in plasma rGH levels being elevated to 100-400 times normal levels (Palmiter *et al.* 1982; Shea *et al.* 1987). GH modulates growth via insulin-like growth factors (somatomedins) that activate receptors in target tissues (Mathews *et al.* 1988). Inheritance of the block of rGH 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.

Of the 40 animals studied, 20 were heterozygously transgenic and 20 lacked transgenes and served as normal controls. Transgenic animals were easily differentiated by their larger size at 28 days of age. Only males were used in order to avoid variation in feeding, behaviour, and mass associated with female estrous cycles.

2.7.2 *General Rearing Conditions*

All mice were obtained from breeding colonies maintained at McMaster University. Breeding groups (1 male mated to 2 females) were housed in clear plastic cages (length ×

width × height = 27 × 21 × 15.5 cm) at 22 ± 2°C with a photoperiod of 12 h light : 12 h dark. A stainless steel hopper, secured 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 5 days. Each container was fitted with a polyester air filter to protect against airborne infections. All protocols were consistent with the guidelines of the Canadian Council on Animal Care.

2.7.3 *Dry Mass Budget Containers*

Mice were preweighed and placed individually in cages modified to allow measurement of mass budgets. Males were separated at weaning to prevent aggressive interactions. For experiments, regular breeding cages were modified as follows. (*i*) Bedding material was removed and replaced by a raised steel grid floor (7-mm^2 mesh). Faeces fell through this grid, preventing coprophagy. *(ii)* Rather than providing food in the hopper, 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. The oblong pellets were arranged as a bundle so mice could not directly contact the adhesive. Each dish was fastened within a 9 cm diameter plastic petri dish using 18 gauge copper wire, 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 had fully cured. Dishes were removed before the mice ate enough to encounter the silicone. Mice were habituated to the experimental containers for 2 days before the mass budget measurements began.

2.7.4 *Variables Investigated*

Dry mass budgets for each mouse were performed over 11 consecutive days (ages 50 -61 days). Each day mice were weighed, data recorded, cages cleaned and disinfected, and water and food replenished. Growth was calculated as the gain of live wet mass between the beginning and end of the 11-day interval. Five transgenic and five normal males were sacrificed and oven-dried to constant mass at 60°C to provide a wet-mass to dry-mass conversion factor. To minimize variation associated with gut content, mice were fasted for 11 h prior to being killed. There was no difference in water content between strains, and the conversion factor (0.32 ± 0.01 (SE)) was similar to that obtained for mice by others (Calvert *et al.* 1986; Searle *et al.* 1992). Unless otherwise stated, all values reported here are in dry mass units.

Five preweighed dishes of food were also dried to constant mass and reweighed to obtain their water content. This conversion factor (0.939 ± 0.001 (SE)) was used to estimate the dry mass of food presented to the mice from its known wet mass. Partially consumed food pellets and crumbs were collected, weighed, and replaced with fresh food dishes daily. Consumption was calculated by subtracting the dried mass of uneaten food from the estimated dry mass provided initially. Faeces were also collected daily, oven-dried to constant mass at 60°C, and weighed.

The following variables were considered: dry mass gained (mg), G; dry mass of food consumed (mg), C; dry mass of faeces deposited (mg), F; dry mass of food assimilated (mg), A ($A = C - F$); and dry mass of food respired (mg), R ($R = C - G - F$). Individual rates, expressed in milligrams mass per milligram dry body mass, were calculated as averages of the

11-day period and then converted to daily measures.

Food utilization and conversion efficiencies were calculated as: assimilation (or digestive) efficiency (%), *AE* ($AE = [(C - F) \div C] \times 100\%$); gross production efficiency (%), *GPE* ($GPE = (G \div C) \times 100\%$); and net production efficiency (%), *NPE* ($NPE = [G \div (C - F)] \times 100\%$) (Timon *et al.* 1970; Sutherland *et al.* 1974).

2.7.5 *Analytical Methods*

Mass budgets were calculated in milligrams dry mass per milligram dry body mass per day to eliminate body size differences between normal and transgenic animals. Overall means (± SE) were calculated for each variable. To facilitate comparisons, transgenic results were also expressed as a percentage of respective normal values. Statistical analyses consisted of *t*-tests. Data from both groups were analysed individually and from the two groups as a pooled sample, by regression analysis.

2.8 RESULTS

The dry mass budgets for normal and transgenic mice are provided in Table 2.1 and reveal the impact of genetically engineered growth enhancement on physiological resource allocation.

2.8.1 *Body Mass and Growth*

Supermice were 153% heavier than normal mice (12.13 ± 0.16 versus 7.91 ± 0.14 g, $p <$ 0.0005) and their growth rates were 220% that of normal mice (0.011 ± 0.001 versus 0.005 ± 0.001 mg / mg dry body mass per day, $p < 0.0005$, during the study period (Fig. 2.1)).

2.8.2 *Consumption*

Mass-specific consumption rates of transgenic mice were 6% less than normal mice (0.47 ± 0.01 and 0.50 ± 0.01 mg / mg dry body mass per day, respectively, $p < 0.025$), despite their increased costs of growth (Table 2.1). Regression analysis on pooled data showed no significant relationship between mass-specific consumption rate and growth rate. Within groups, however, consumption increased with greater growth rates (Fig. 2.2), and this was statistically significant for Supermice ($p < 0.02$).

2.8.3 *Faeces*

The mass-specific rate of faecal deposition of Supermice was 102% that of normal males (0.169 ± 0.004 versus 0.166 ± 0.003 mg faeces / mg dry body mass per day, ns).

2.8.4 *Assimilation Efficiency and Assimilation Rate*

Assimilation efficiency was slightly less in Supermice than in normals (64.1 ± 0.6 versus 66.7 ± 0.8%, $p < 0.025$). The combination of reduced feeding rates and reduced *AE* resulted in Supermice having an assimilation rate 9% less than normal mice (0.30 ± 0.01 versus 0.33 ± 0.01 mg assimilate / mg dry body mass per day, $p < 0.005$).

2.8.5 *Respiration*

The mass-specific respiration rate of Supermice was 11% less than that of normal mice (0.29 ± 0.01 and 0.33 ± 0.01 mg / mg dry body mass per day, $p < 0.0005$).

2.8.6 *Gross and Net Production Efficiency*

Supermice were more than twice as efficient at converting ingested food into body mass than normal mice (*GPE* = 2.5 ± 0.1 versus 1.1 ± 0.1%, respectively, $p < 0.0005$). Similarly, the efficiency of converting digested (assimilated) food into transgenic body tissue was 244% greater than normal conversion efficiency (*NPE* = 3.9 ± 0.2 versus 1.6 ± 0.1%, , $p < 0.005$). Enhanced transgenic growth was attained entirely by increased food conversion efficiency rather than by increased feeding (Figs. 2.3 and 2.4). Regression analyses revealed strong relationships between gross or net production efficiencies, and mass-specific growth rates for both individual and pooled data (Normal *GPE*: $r^2 = 0.96$, Transgenic *GPE*: $r^2 = 0.93$, Pooled *GPE*: $r^2 = 0.97$; and Normal *NPE*: $r^2 = 0.95$, Transgenic *NPE*: $r^2 = 0.90$, and Pooled *NPE*: $r^2 = 0.95$). Remarkably, the transgenic regression lines were simple extrapolations of those obtained for normal mice.

2.9 DISCUSSION

The Supermouse provides a valuable model for evaluating the impact of genetically engineered growth enhancement in vertebrates (Table 2.1). Artificial selection or mutations resulting in large, rapidly growing mice yield characteristics similar to those observed in Supermice, including lethargy, reduced longevity, and infertility (MacArthur 1949; Falconer 1953; Eklund and Bradford 1977; Goodrick 1977; McCarthy 1980). Such selection commonly yields improved production efficiencies like those obtained here (Malik 1984), although increased feeding rates sometimes result as well (McCarthy 1980; Roberts 1981). Growth enhancement in other transgenic GH strains of mice is also associated with changes similar to those reported here (Palmiter *et al.* 1982, 1983; Hammer *et al.* 1985 *a*, *b*; Brem *et al.* 1989; Knapp *et al.* 1993; Wolf *et al.* 1993). This is consistent with results of Pidduck and Falconer (1978) showing that the main feature of mice modified by artificial selection or mutations affecting body size is GH.

During the experimental period, the body mass of Supermice was 153% that of normal mice ($p < 0.0005$). The mean maximal size of Supermice, on a lifetime basis, was 57.5 ± 0.7 (SE) g, (n = 24) compared to 33.5 ± 0.8 (SE) g, (n = 24) for normal males ($p < 0.05$; C. D. Rollo, unpublished data). This represents a 171% difference. Growth enhancement varies widely among transgenic GH strains. For example, ovine GH mice obtained only a 32% increase (Pomp *et al.* 1992). Although human, bovine, ovine, porcine, and rat GH were originally considered to be functionally equivalent (Hammer *et al.* 1985*a*), notable differences may arise in association with regulatory sequences in flanking regions of these genes

(Stefaneanu *et al.* 1993; Wolf *et al.* 1993). Rat GH definitely has comparable activity and structure to endogenous mouse GH (Schindler *et al.* 1972). Differences among strains may stem from variation in the number or site of incorporation of transgenes. Our mice appear to be simply large versions of normal mice, but transgenic strains with other promoters may be sterile or express spontaneous lactation (Bchini *et al.* 1991; Steger *et al.* 1991; Milton *et al.* 1992).

Supermice eat more than normal mice simply because they are larger, but when considered on a mass-specific basis, feeding rate was clearly depressed despite the imposition of a twofold increase in growth costs (Table 2.1). Failure to increase feeding rates was observed in other transgenic GH animals as well (Etherton *et al.* 1987; Campbell *et al.* 1989; Pursel *et al.* 1989; Pomp *et al.* 1992). Several authors proposed that feeding regulation may involve an interaction of GH or its antagonist, somatostatin with targets in the hypothalamus (Wade 1974; Hall *et al.* 1986). High expression of transgenic GH genes suppresses production of endogenous GH in the pituitary (Palmiter *et al.* 1983; Miller *et al.* 1989; Stefaneanu *et al.* 1993). The metallothionein promoter is mainly activated in the liver or inappropriate cells in the pituitary (Bremner and Beattie 1990; Stefaneanu *et al.* 1993). If the regulatory mechanism linking consumption to growth rates resides in the pituitary (Anand and Brobeck 1951), then transgenic GH expressed in inappropriate tissues could lead to maladaptive suppression of appetite by negative feedback on production of endogenous GH.

Although disruption of feeding regulation may explain the failure of Supermice to match feeding rates to costs (which could be modified by an appropriate promoter), the fact that their mass-specific feeding rates are very similar to normal animals suggests that

regulation of feeding may instead target body mass itself. Under dietary restriction, normal mice also maintain similar mass-specific rates of consumption and metabolism while adjusting body size to resource levels (McCarter *et al.* 1985; Duffy *et al.* 1989; Masoro 1993).

Dietary restriction in rodents is associated with a reorganization of resource allocation that involves decreased body size, decreased levels of circulating GH, reduced or altered patterns of thermogenesis, and reduced reproductive rates. In addition, immunological functions, longevity, and the period of reproductive competence are greatly extended (Holehan and Merry 1986; Igram and Reynolds 1987; Weindruch and Walford 1988; Holliday 1989; Mounier *et al.* 1989; Finch 1990; Lachmansingh and Rollo 1994). With *ad libitum* food, growth and reproduction are enhanced, but longevity is reduced. Within the normal size limits of mice, larger body sizes or selection for faster growth also increases fecundity (Falconer 1953; Bradford *et al.* 1980; Bronson 1984). Fecundity then declines, however, with further accentuation of size (Falconer and King 1953). The reaction norm to dietary restriction may serve to adaptively defer reproduction under stress (Holliday 1989; Lachmansingh and Rollo 1994). Supermice may be victims of this reaction norm, which may be partially modulated by GH as a key endocrine signal. Excessive growth superimposed on this norm would be expected to maintain relatively unaltered mass-specific rates of metabolism and feeding. Maladaptation arises because the system has no evolutionary experience in accommodating unregulated rapid growth.

Transgenic and normal mice exhibit similar compensatory elevations in feeding rates following periods of food deprivation (L. N. DelCotto and C. D. Rollo, unpublished data). Thus, undereating in Supermice is not due to limited digestive capacity. Figure 2.2 is of

particular relevance to interpretations of possible life-history trade-offs. The fact that resource intake did not increase across strains means that trade-offs can be detected by comparing normal and transgenic mice. Within strains, however, feeding rates do vary with growth rates, a fact that could obscure trade-offs within treatments. Thus, the only comparison really relevant to the mouse trade-off structure is across strains. Many studies have dismissed consistent across-treatment trends (such as decreased body size and enhanced longevity under dietary restriction), because within treatments, neutral or opposite trends may occur (e.g. the largest restricted rodents may live longer, see Igram and Reynolds 1987). Such results could simply reflect variation of fitness within groups, with higher quality mice being generally superior.

Even in normal mice, the maximal sustainable elevation of metabolism or consumption levels is no more than double, and maximal levels (which can be achieved only briefly) are no more than 4.5 times basal (Lachmansingh and Rollo 1994). Because Supermice show doubled costs without elevating feeding, reductions in other processes are necessary. Hoffmann and Parsons (1991) and Parsons (1991) found that organisms exhibit a relatively universal reduction in respiratory expenditures under chronic stress. Indeed, respiration, as measured by the mass budget, shows a significant reduction in Supermice (Table 2.1). Supermice, by virtue of their excessive growth and reduced feeding, may operate in a self-imposed state of stress. Clearly, any adjustments reducing respiratory costs or enhancing feeding would allow more energy to be channelled into growth, or to be reallocated to restore compromised functions such as behaviour, fertility, or longevity assurance systems.

Although the difference was significant, the *AE* of Supermice was only 2% less than

that of normal animals (Table 2.1). Changes in *AE* have never proven important in selection for growth rate in mice (McCarthy 1980). Supermice, however, were significantly more efficient in converting food into tissue than were normal males (Table 2.1). Improved conversion efficiencies have been consistently obtained in animals artificially selected for large size, injected with GH, or genetically engineered for enhanced growth (e.g. Timon *et al.* 1970; Roberts *et al.* 1980; Malik 1984; Bernier *et al.* 1986; Grosbeck *et al.* 1987; McCarthy and Roberts 1989; Pursel *et al.* 1989; Bootland *et al.* 1991; Wolf *et al.* 1991; Pomp *et al.* 1992).

This study coincided with the late moderate phase of growth of the mice, which explains the relatively low values of gross and net production efficiencies (Table 2.1). Greater conversion efficiencies were obtained in younger, faster growing mice (L. J. Kajiura and C. D. Rollo, unpublished data). Gains in body mass represent only a fraction of the energy needed to produce them (Millward *et al.* 1976), so the costs to Supermice undoubtedly exceed realized growth. When data for normal and transgenic mice were pooled, it was evident that Supermice represented an extension of a fundamental strategy relating growth rates to production efficiencies that is expressed in normal mice (Figs. 2.3 and 2.4), supporting the hypothesis that the Supermouse represents a non-adaptive extension of an adaptive strategy reflected in the reaction norms and genetic correlation structure of normal mice.

Although thermogenesis in Supermice was not reduced (C. D. Rollo, unpublished data), behaviour, longevity, and reproduction, were compromised, suggesting that improved efficiencies were obtained by robbing other resource sinks. From this perspective, the

"improvement" in growth and production efficiency may better be characterized as physiological "stress". Transgenic GH varieties may be victims of an energy crisis resulting from increased costs with no offsetting resource elevations. Despite possessing enhanced abilities to convert food into body tissue, Supermice exhibit several shortcomings. Mean maximal longevity of normal males (790.7 days, n = 6) and normal females (659.8 days, n = 26) was more than double that of transgenic males (347.5 days, n = 55) and transgenic females (303.3 days, n = 71). Differences between the strains were all significant ($p < 0.05$) (C. D. Rollo, unpublished data). If Supermice represent a maladaptive extension of the reaction norm exhibited under dietary restriction, decreased longevity might arise through compromised investment in antioxidant defense or DNA and protein repair or replacement systems (Weindruch and Walford 1988; Holliday 1989; Masoro 1993). Pathological symptoms are generally consistent with accelerated rates of aging (Steger *et al.* 1993; Wolf *et al.* 1993).

Transgenic GH females have low fertility or are sterile (Hammer *et al.* 1985*a*; Bartke *et al.* 1988; Brem *et al.* 1989; Pursel *et al.* 1990; Naar *et al.* 1991), which equates to "evolutionary failure". Only 45% of Supermice were fertile compared to essentially 100% of normal females. Of those that reproduced, lifetime reproduction of Supermice averaged only 7.7 pups compared to 35.8 for normal mothers ($p < 0.05$) (C. D. Rollo, unpublished data). Normal rodents suppress reproduction under stress, and the fact that progesterone can restore reproduction in transgenic human GH mice (Bartke *et al.* 1988), suggests that Supermice may have hormonally blocked reproduction.

Lachmansingh and Rollo (1994) document that activity in Supermice is greatly

reduced, particularly for expensive behaviours like wheel running. Supermice obtained considerable energetic savings by sleeping 3.4 h / day more than normal mice. The docility and lethargy of transgenic animals would likely compromise foraging and avoidance of predators in nature. Similarly, trade-offs related to elevated growth through increased metabolic efficiency would likely constrain natural mouse populations from attaining very large sizes in the same way.

Falconer (1977) postulated that size is an evolutionary compromise among conflicting fitness features (e.g. small mice are less fecund, but large mice are more prone to predation). In normal strains, maximal metabolic rates and longevities may occur at intermediate sizes and such mice may also have a greater degree of metabolic scope (Sacher and Duffy 1979). Roberts (1981) and Derting (1989) showed that rodents with elevated growth or metabolic rates have compromised performance under dietary restriction even though they are superior to controls on adequate diets. Thus, selection in variable environments may select phenotypes that are "safely tuned" to resource levels. A reaction norm may vary the mean phenotype across this range, emphasizing high reproduction, high production efficiency, and lower longevity assurance in rich environments, and decreased production and enhanced longevity in resource-poor habitats. The fact that Supermice express doubled growth rates, and that both normal and transgenic mice are not limited by digestive capacity emphasizes that neither of these variables have been maximized by selection contrary to optimality hypotheses (Maynard Smith 1978; Sibly and Calow 1983; Pyke 1984).

Genetic correlations associated with bidirectional selection for body size share remarkable similarities with the plastic norm modulated by dietary restriction within the

normal size range of mice. Mice resulting from extreme directional selection and genetically engineered GH mice have common maladaptive syndromes, suggesting that they represent disruption of a coadaptive balance in normal animals, associated with extensions of evolved norms and genetic correlation structures beyond their normal ranges.

The results of this study may have practical application for attempts to derive functional transgenic GH livestock. Studies in progress indicate that dietary alterations can restore some compromised functions (e.g. behavioural activity restored by high carbohydrate diets). Crossing transgenic GH strains to hyperphagic strains may also be a worthwhile avenue for increasing food intake, and alleviating the impact of growth costs on other aspects of physiological resource allocation, such as reproduction or longevity assurance systems.

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TABLE 2.1. Comparison of means and SE for various measures of the dry mass budget of transgenic Supermice and normal male *Mus musculus* aged 50-61 days.

	Normal		Transgenic		
	MEAN	SE	MEAN	SE	*t*-test
Body Mass (g)	7.91	0.14	12.13 (153)	0.16	$p < 0.0005$
Growth Rate (mg/mg dry body mass per day)	0.005	0.001	0.011 (220)	0.001	$p < 0.0005$
Consumption Rate (mg/mg dry body mass per day)	0.499	0.008	0.471 (94)	0.008	$p < 0.025$
Faecal Rate (mg/mg dry body mass per day)	0.166	0.003	0.169 (102)	0.004	ns
Assimilation Rate (mg/mg dry body mass per day)	0.333	0.007	0.303 (91)	0.007	$p < 0.005$
Respiration Rate (mg/mg dry body mass per day)	0.328	0.007	0.291 (89)	0.006	$p < 0.0005$
Assimilation Efficiency (%)	66.7	0.8	64.1 (96)	0.6	$p < 0.025$
Gross Production Efficiency (%)	1.1	0.1	2.5 (227)	0.1	$p < 0.0005$
Net Production Efficiency (%)	1.6	0.1	3.9 (244)	0.2	$p < 0.0005$

NOTE: Probabilities were determined by *t*-tests. Each group consisted of 20 mice. Numbers in parentheses are the values for the transgenic mice expressed as a percentage of the values for the normal mice.

FIG. 2.1 Growth of normal (■) and transgenic (□) male *Mus musculus*. Equations for the regression lines are as follows: **Normal**: $BODY\ MASS = 0.045(AGE) + 5.417$, $r^2 = 0.946$, df = 10, $p < 0.0001$; **Transgenic**: $BODY\ MASS = 0.149(AGE) + 3.933$, $r^2 = 0.988$, df = 10, $p < 0.0001$.

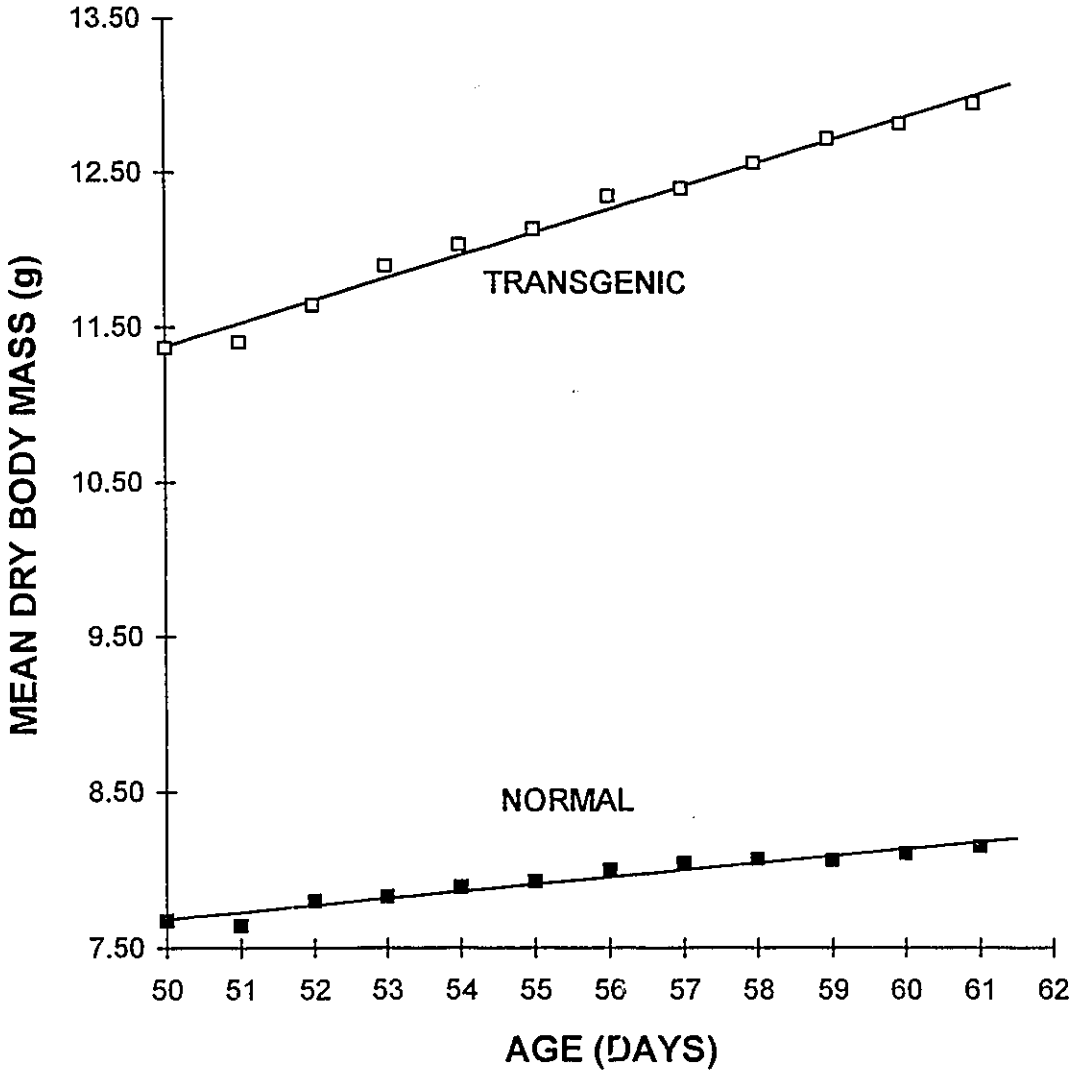

FIG. 2.1 KAJIURA, L. J., and ROLLO, C. D. 1994.

FIG. 2.2. Relationship between mass-specific rates of growth (GR) and consumption (CR) of normal (■) and transgenic (□) male *Mus musculus*. Equations for the regression lines are as follows: Normal, $CR = 3.525(GR) + 0.478$, $r^2 = 0.052$, df = 18, $p > 0.30$; Transgenic, $CR = 5.186(GR) + 0.409$, $r^2 = 0.283$, df = 18, $p < 0.02$; Normal and Transgenic (pooled), $CR = 0.059(GR) + 0.485$, $r^2 = 4.747 \cdot 10^{-3}$, df = 38, $p > 0.50$.

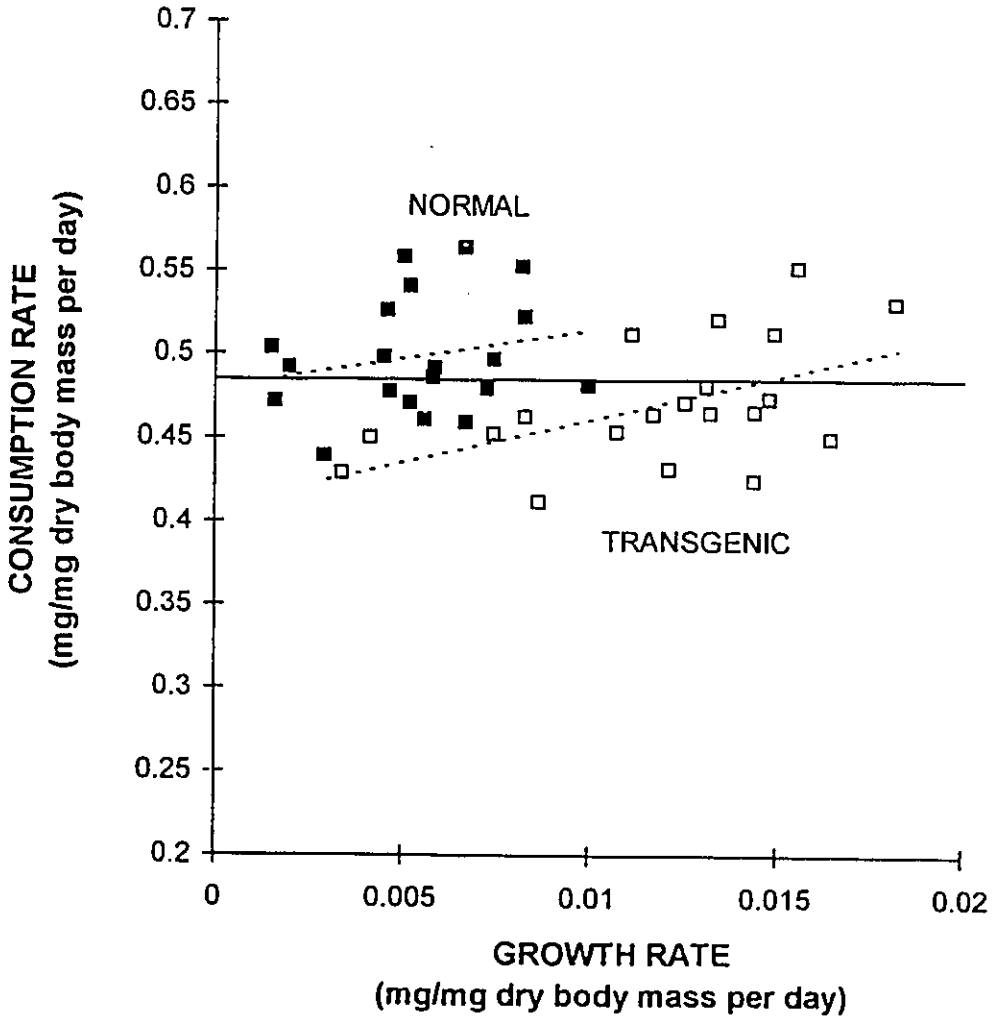

FIG 2.2 KAJIURA, L. J., and ROLLO, C. D. 1994.

FIG. 2.3 Relationship between gross production efficiency (*GPE*) and mass-specific growth rate (*GR*) of normal (■) and transgenic (□) male *Mus musculus*. Equations for the regression lines are as follows: **Normal**: $GPE = 195.649(GR) + 0.024$, $r^2 = 0.970$, df = 18, $p < 0.0001$; **Transgenic**: $GPE = 190.124(GR) + 0.246$, $r^2 = 0.932$, df = 18, $p < 0.0001$; **Normal and Transgenic (pooled)**: $GPE = 206.192(GR) + 0.010$, $r^2 = 0.971$, df = 38, $p < 0.0001$.

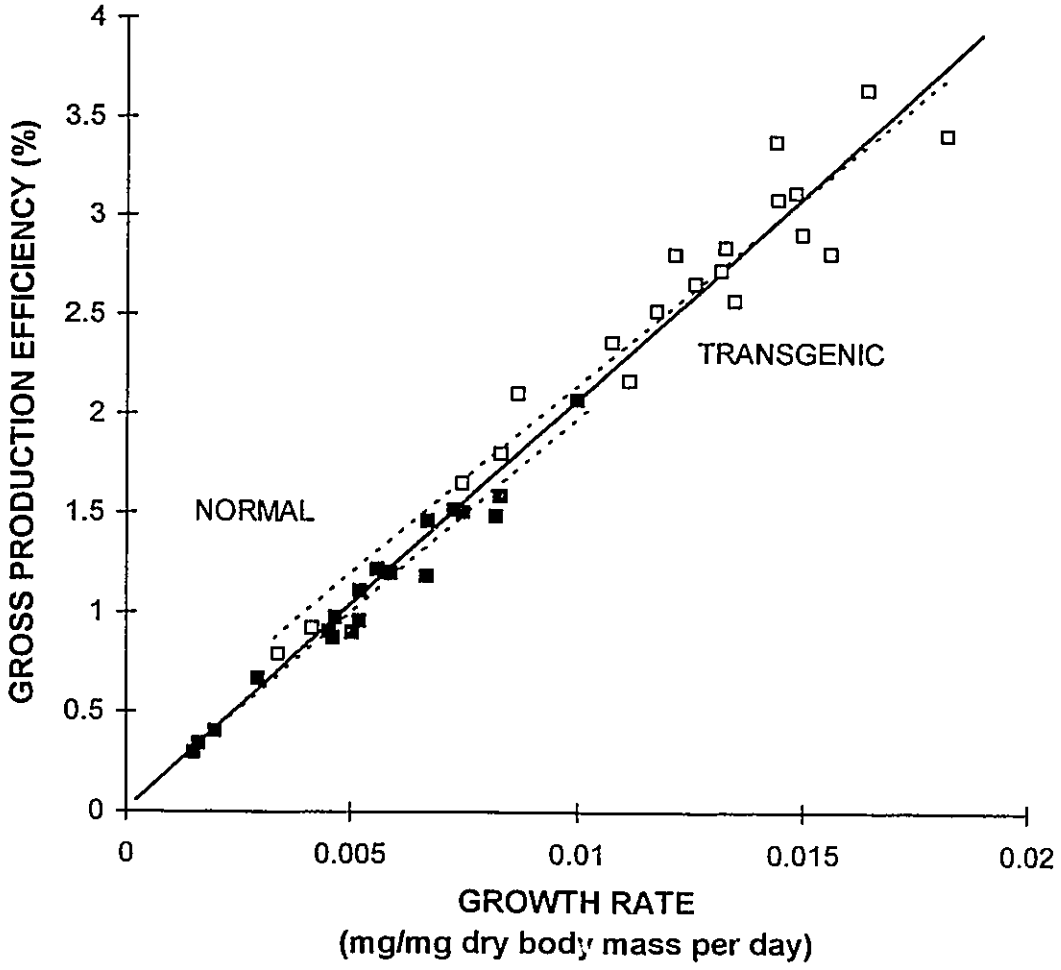

FIG. 2.3 KAJIURA, L. J., and ROLLO, C. D.1994.

FIG. 2.4 Relationship between net production efficiency (*NPE*) and mass-specific growth rate (*GR*) of normal (■) and transgenic (□) male *Mus musculus*. Equations for the regression lines are as follows: **Normal** : $NPE = 279.326\ (GR) + 0.097$, $r^2 = 0.954$, df = 18, $p < 0.0001$; **Transgenic**: $NPE = 284.476\ (GR) + 0.518$, $r^2 = 0.909$, df = 18, $p < 0.0001$; **Normal and Transgenic (pooled)**: $NPE = 320.013(GR) - 0.016$, $r^2 = 0.957$, df = 38, $p < 0.0001$.

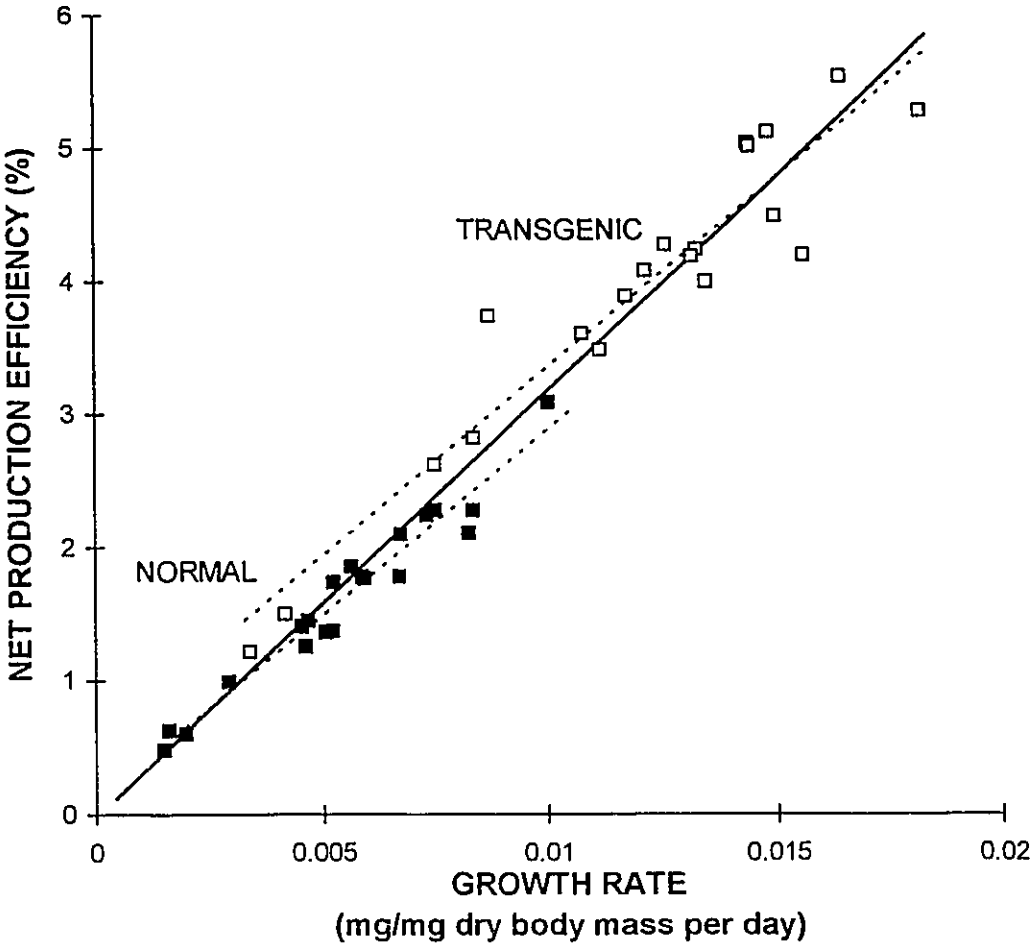

FIG. 2.4 KAJIURA, L.J., and ROLLO, C. D.1994.

SECTION III

THE ONTOGENY OF RESOURCE ALLOCATION IN GIANT TRANSGENIC RAT GROWTH HORMONE MICE

3

The ontogeny of resource allocation in giant transgenic rat growth hormone mice.

(Kajiura, L. J., and Rollo, C. D. 1995. A manuscript prepared for publication in the Canadian Journal of Zoology).

3.1 RATIONALE AND OBJECTIVES for Kajiura and Rollo (1995)

This manuscript explores the ontogenic changes in growth, feeding, and resource allocation of Supermice, transgenic for extra copies of rat growth hormone fusion genes. Although results were analysed using methods similar to that of Kajiura and Rollo (1994), all data presented herein are completely new observations for transgenic and normal mice during both their early and late growth periods (mice aged 25-40 days old, and 47-62 days old, respectively). By examining the ontogenic changes in feeding and overall physiological resource allocation of normal and transgenic mice, the present study represents what we believe is the first detailed ontogenic comparison of transgenic GH animals with their non-transgenic normal relatives. The late growth phase encompasses a similar time interval to that period monitored in Kajiura and Rollo (1994) and thus represents an independent verification of previous results. Our findings will be of practical interest to those engineering transgenic organisms and also offers several significant theoretical insights relevant to life-history evolution.

3.2 CLARIFICATION OF CONTRIBUTIONS for Kajiura and Rollo (1995)

Results from Kajiura and Rollo (1994) suggested that an ontogenic assessment of the changes in resource allocation for transgenic Supermice and normal mice would be a worthwhile endeavour. Mass budgets for 20 normal and 20 transgenic male mice were monitored for two 15 day periods, over a 192 consecutive day period. During the course of this project, I also aided in the collection of life-history data (longevity, growth, and reproduction) for both transgenic and normal mice.

My contributions were as follows:

1. **Intellectual Contributions**:

   (*i*) Review of the scientific literature on transgenic GH animals, specifically on physiological resource allocation, was conducted largely by myself.

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

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

   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. These observations provided longevity data for each

of the two strains of mice. Injured mice or mice displaying unusual health symptoms were isolated from the main breeding colony pending further medical diagnosis.

(*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, diet treatment, number of offspring, and offspring body mass were recorded to compile data on the reproduction for transgenic and normal strains. At 20 d of age, mice were weaned to form new breeding groups or to be used for experimental purposes.

(*iii*) All animals maintained in the breeding colony were weighed weekly to accumulate growth data for both transgenic and normal animals.

Food:

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

Water:

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

Animal Breeding Cages:

Cages, which contained 1 male and 2 females were washed, disinfected, and bedding material replaced every 5 days. Cages ,which held five adult mice, were cleaned at 2 day intervals.

3. **Maintenance of Animal Quarters**:

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

4. **Experimental Design, Preparation, Data Documentation, and Statistical Analyses for Mass Budget Study**:

(*i*) Preparation, construction, and assembly of general rearing containers and experimental mass budget containers (see APPENDIX B).

(*ii*) Preparation, assembly, and weighing of food pellet dishes for daily measurements of food consumption. Construction and weighing of faecal collection vessels for daily deposition measures.

(*iii*) Documentation and calculation of food consumption, faecal deposition, growth, assimilation, assimilation efficiency, respiration, gross production efficiency,and net production efficiency. Statistical comparisons (*t*-tests, correlation-regression analyses) were conducted using Excel, Version 5 (Microsoft® Inc.), in consultation with C. D. Rollo.

3.3

The ontogeny of resource allocation in giant transgenic rat growth hormone mice

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KAJIURA, L. J., and ROLLO, C. D. 1995. The ontogeny of resource allocation in giant transgenic rat growth hormone mice.

3.4 ABSTRACT

Dry mass budgets were conducted on transgenic metallothionein-1 rat growth hormone mice and normal *Mus musculus* to assess ontogenic changes in growth, feeding, and resource allocation. Younger mice had higher rates and efficiencies of growth than older mice. Young transgenic mice and normals were relatively similar for most features but became progressively dissimilar with time. Transgenic mice never grew faster than the most rapid growth observed in normal mice, but grew larger by maintaining higher growth rates into later ages. On a mass-specific basis, transgenic animals consumed less food than normals. Reduced feeding was not simply a reflection of the allometric scaling of food intake with larger body size, as younger transgenic mice ate less food than normals of equivalent size even on an absolute basis. Transgenics achieved increased growth via superior production efficiency and ontogenically, by maintaining greater efficiency into later ages. Differences in feeding and efficiency were detectable even before the mice diverged much in size. A unitary relationship relating production efficiencies and growth rates for the older mice was confirmed, but younger transgenics and normal controls displayed fundamentally different relationships between efficiencies and rates of growth. Insights into growth regulation, feeding, life-history trade-offs, and allometric theory are discussed.

3.5 INTRODUCTION

Biotechnology permits specific redesign of naturally evolved genomes and provides ideal probes for assessing the organization and integration of processes and features comprising a species' adaptive suite. Transgenesis exaggerates or represses one attribute and disrupts its coadaptation with others, thus providing insight into naturally evolved coadaptation.

Acquisition and processing of resources are limited so negative trade-offs are expected among conflicting demands that contribute to fitness. Amplification of one attribute must ultimately impact negatively on others, although short-term elevations in feeding, increased efficiencies, or use of stores may offset immediate costs (Tuomi *et al.* 1983). Such trade-offs are central to the "principle of allocation", an underlying assumption of life-history theory (Sibly and Calow 1986; Lessells 1991; Stearns 1992; Roff 1993; Rollo 1994). Naturally evolved animals reflect an adaptive balance of investments among competing demands obtained via endocrine regulation (i.e. growth, reproduction, defense, behaviour, thermogenesis). Transgenic metallothionein-1 rat growth hormone mice display adult body sizes and growth rates almost twice that of their normal relatives. They possess a single genetic alteration, multiple copies of rat growth hormone (rGH) genes incorporated into one chromosome (Palmiter *et al.* 1982; 1983). Transgenic mice, in their late growth stage, do not elevate specific feeding rates to pay the costs of accelerated growth (Kajiura and Rollo 1994), so the emergence of negative trade-offs among key fitness features becomes unavoidable. Since growth rates and body sizes are strongly correlated with most life-history and

physiological attributes (Calder 1984; Rollo 1994), giant transgenic rat growth hormone mice provide a particularly powerful model for evolutionary ecology.

Mice display several distinct phases of growth. Kajiura and Rollo (1994) considered the late moderate phase of growth, when mice (aged 50-61 days old) expressed relatively low production efficiencies. The present study evaluates the impacts of enhanced growth during ontogeny by comparing how transgenic mice and normal controls allocate resources during both early and late growth stages. In particular, we reasoned that much could be learned by examining the early period when growth is most rapid. What differences are apparent between the mice before size diverges greatly? Here we present an independent replication of our earlier study (Kajiura and Rollo 1994) that confirms the stability of the initial results in this transgenic system, and we compare these new results to rapidly growing younger mice aged 25-40 days old. The "transgenic correlation structure" revealed by giant rat growth hormone mice, provides information relevant to applied agricultural studies in growth enhancement, and we explore insights into naturally evolved coadaptive integration relevant to evolutionary ecology generally.

3.6 METHODS

3.6.1 *Animals Used*

Transgenic rat growth hormone mice (strain Tg[MT-1, rGH], Bri2) were obtained from a large breeding colony established at McMaster University. Palmiter *et al.* (1982) engineered this strain via microinjection of fusion genes (metallothionein-1 promoters fused to rat growth hormone structural genes) into the pronuclei of fertilized mouse eggs. Transgenic mice possess tandem rGH genes incorporated into a single chromosome, producing elevations of plasma rGH 100 to 400 times normal (Palmiter *et al.* 1982; Shea *et al.* 1987). GH-modulated production of insulin-like growth factors (somatomedins), activates receptors promoting proliferation of tissues such as cartilage, bone, and muscle (Mathews *et al.* 1988; Thissen *et al.* 1994). The rGH genes are inherited as a Mendelian block so that equal numbers of transgenic and normal control animals are generated when heterozygously transgenic males are mated to normal females. This allows control of genetic background.

Of the 40 animals studied, 20 were heterozygously transgenic and 20 lacked transgenes and served as normal controls. Transgenic animals were readily distinguished by their larger mass at 28 days of age. Only males were studied to avoid variation associated with female reproductive cycles.

3.6.2 *General Rearing Conditions*

Breeding groups (1 male and 2 females) were housed in clear plastic cages (length × width × height = 27 × 21 × 15.5 cm) at 22 ± 2°C with a photoperiod of 12 h light: 12 h dark.

A stainless steel hopper, secured over each container, supported food pellets and a water bottle. All animals were fed *ad libitum* (Lab Diet®, No. 5001, PMI Feeds Inc., St. Louis, Missouri). Cages were bedded with Beta-Chip® (Northeastern Products Corp., Warrensburg, New York) and cleaned every 5 days. Each container was fitted with a polyester filter to protect against possible airborne infections. All protocols were consistent with the guidelines of the Canadian Council on Animal Care.

3.6.3 *Dry Mass Budget Containers*

Mice were preweighed and placed individually in cages modified to measure mass budgets. Regular breeding cages were modified as follows: (*i*) bedding material was removed and replaced by a raised steel grid floor (7-mm^2 mesh). Faeces fell through this grid, preventing coprophagy. (*ii*) Rather than providing food in the hopper, 11 preweighed pellets were secured to the bottom of a 5.3 cm diameter plastic petri dish with translucent Silicone II Sealant® (General Electric Inc., Toronto, Canada). The oblong pellets were arranged as a bundle so that the mice could not directly contact the adhesive. Each dish was fastened within a 9 cm diameter petri dish using 18 gauge copper wire, 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 had fully cured, and were removed before mice ate enough to encounter the silicone. Mice were habituated to the experimental cages for 2 days before commencing the mass budget. Cages were cleaned and disinfected daily.

3.6.4 *Variables Investigated*

Dry mass budgets for each mouse were obtained over two 15 day intervals (early growth: mice aged 25-40 d, and late growth: mice aged 47-62 d). Growth was measured as gain of live wet mass measured at the beginning and end of each 15 d interval. Ten normal and 10 transgenic male mice (62 d old), and 10 normal and 10 transgenic males (40 d old) were sacrificed and oven-dried to constant mass at 60°C to provide a wet-mass to dry-mass conversion factor. To minimize variation associated with gut contents, mice were fasted for 11 h before being sacrificed. There was no difference in water content between strains, however, differences were found between growth phases ($p < 0.0008$). Wet-mass to dry-mass conversion factors were 0.267 ± 0.004 (SE) and 0.294 ± 0.005 (SE) for younger and older mice, respectively. Unless otherwise stated, all measurements reported are in dry mass units.

Five preweighed dishes of food were also dried to constant mass and reweighed to determine their water content. This conversion factor (0.939 ± 0.001 (SE)) was employed to estimate the initial dry mass of food presented to the mice. Crumbs and partially consumed pellets were collected, weighed, and replaced daily with fresh food dishes. Consumption was calculated by subtracting the dried mass of uneaten food from the estimated dry mass provided initially. Faeces were also collected daily, oven-dried to constant mass at 60°C, and weighed.

The following variables were considered: dry mass gained (mg), G; dry mass of food consumed (mg), C; dry mass of faeces deposited (mg), F; dry mass of food assimilated (mg), A ($A = C - F$); and dry mass of food respired (mg), R ($R = C - G - F$). Individual rates, expressed in units of milligrams dry mass per milligram dry body mass, were calculated as

averages for each 15 d period and then converted to the daily measures.

Food utilization and conversion efficiencies were calculated as: assimilation (or digestive) efficiency (%), *AE* ($AE = [(C - F) \div C] \times 100\%$); gross production efficiency (%), *GPE* ($GPE = (G \div C) \times 100\%$); and net production efficiency (%), *NPE* ($NPE = [G \div (C - F)] \times 100\%$).

3.6.5 *Analytical Methods*

Mass budgets were calculated in units of milligram dry mass per milligram dry body mass per day to eliminate body mass differences between normal and transgenic animals. Overall means (± standard errors) were calculated for each yield variable and these were analysed with *t*-tests. Transgenic results were also expressed as a percentage of respective normal values. Data from both groups were also analysed by correlation-regression analysis.

3.7 RESULTS

Dry mass budgets for the early and late growth phases of normal and transgenic mice are presented in Table 3.1. As expected, much higher rates and efficiencies were obtained during early growth compared to later growth stages.

3.7.1 *Body Mass and Growth*

Transgenic mice were only 129% heavier than normal mice (5.81 ± 0.17 g versus 4.52 ± 0.09 g, $p < 0.0005$) during the early growth period (Fig. 3.1) and the mass-specific growth rates of transgenic mice (0.045 ± 0.002 mg / mg dry body mass per day) were not statistically resolved from normals (0.044 ± 0.002 mg / mg dry body mass per day). Transgenics were 148% heavier than normal mice during later growth (10.58 ± 0.17 g versus 7.14 ± 0.08 g, $p < 0.0005$, Fig. 3.1) when mass-specific growth rates of transgenics were 183% that of normals (0.011 ± 0.0005 mg / mg dry body mass per day versus 0.006 ± 0.0003 mg / mg dry body mass per day, respectively, $p < 0.0005$). Fig. 3.1 illustrates regression analyses of age versus mean dry body mass for transgenic and normal mice. Logarithmic transformations were performed on both variables, consistent with standard allometric methodologies. The early growth phase was characterized by rapid growth for both transgenic and normal animals. Growth declined with age, but attenuated much faster in normal mice.

3.7.2 *Consumption*

On an age-specific basis, transgenic mice consumed more food than normal mice

simply because they were larger (Fig. 3.2). Whole-animal consumption rates increased rapidly with age during early growth for both groups but the ontogenic pattern of feeding was strikingly different for the two kinds of mice. Thus, consumption reached a plateau for older normal mice at about 30 d of age even though growth had not ceased at that time. Transgenic mice gradually increased their food intake across the 60 d span of ages. On a mass-specific basis, consumption rates of transgenic mice were 14% less than normals even during early growth (0.746 ± 0.010 mg food / mg dry body mass per day versus 0.868 ± 0.010 mg food / mg dry body mass per day, $p < 0.0005$, respectively) and 8% less than normals during late growth (0.563 ± 0.005 mg food / mg dry body mass per day versus 0.612 ± 0.011 mg food / mg dry body mass per day, respectively, $p < 0.0002$). Transgenic mice displayed lower consumption rates despite their higher growth costs (Figs. 3.3 and 3.4). Figure 3.3 shows that, during the early growth phase, transgenic mice of equivalent body size to normal animals ate less food, even on a whole-animal basis. (i.e. this is not merely an artifact arising from decreased mass-specific consumption associated with larger sizes). This represents a major clarification of the impact of transgenesis on feeding. Mass-specific consumption and respiration rates showed strong positive correlations for both early and late growth phases (Tables 3.2 and 3.3, Fig. 3.5).

3.7.3 *Assimilation Efficiency and Assimilation Rate*

Assimilation efficiency was slightly, but significantly, greater in younger transgenics than in younger normals (66.98 ± 0.46% versus 65.32 ± 0.39%, respectively, $p < 0.008$) while late assimilation efficiency of older transgenics was not significantly different from that of

older normals (65.60 ± 0.54% versus 66.31 ± 0.46%, ns). Assimilation rate of transgenic mice was 12% and 9% less than that of normals in the early and late growth phases, respectively (Table 3.1).

3.7.4 *Respiration*

The early mass-specific respiration rate of transgenic mice was 13% less than that of normal mice (0.455 ± 0.008 mg / mg dry body mass per day versus 0.524 ± 0.007 mg / mg dry body mass per day, respectively, $p < 0.0005$). Late mass-specific respiration rate for transgenics was 10% less than that of normals (0.360 ± 0.005 mg / mg dry body mass per day versus 0.400 ± 0.009 mg / mg dry body mass per day, respectively, $p < 0.0003$). Mass-specific respiration rates and growth rates displayed opposite trends between transgenic and normal mice for both growth phases (Tables 3.2 and 3.3, Fig. 3.6). Figure 3.6 clearly shows, however, that transgenic mice obtained higher growth rates for any given level of respiration (i.e. they are metabolically more efficient).

3.7.5 *Gross and Net Production Efficiency*

Both correlation (Tables 3.2 and 3.3) and regression (Figs. 3.7 and 3.8) analyses revealed strong relationships between conversion efficiencies and mass-specific growth rates. Transgenic mice were more efficient at converting ingested food into body mass than controls during early (transgenic *GPE*: 6.00 ± 0.16%, normal *GPE*: 5.02 ± 0.19%, $p < 0.0003$), and late growth phases (transgenic *GPE*: 1.93 ± 0.08%, normal *GPE*: 1.00 ± 0.06%, $p < 0.0005$). Similarly, the efficiency of conversion of digested food into transgenic body tissue, was

greater than that of normals during early (transgenic *NPE*: 8.96 ± 0.23%, normal *NPE*: 7.70 ± 0.31%, $p < 0.002$) and late growth (transgenic *NPE*: 2.94 ± 0.12%, normal *NPE*: 1.51 ± 0.09%, $p < 0.0005$). When related to mass-specific growth rates, transgenic mice had higher *GPE* and *NPE* than normal mice, and two separate regression lines were resolved during the early phase (Figs. 3.7 and 3.8). During the late growth phase, however, these lines converged into a single relationship. In all cases, a strong positive relationship existed between specific growth rates and production efficiencies. When related to age, production efficiencies were clearly higher in younger faster growing mice, with transgenic mice being slightly more efficient. Production efficiencies fell with age in all mice, but transgenic mice maintained relatively higher production efficiencies into later ages (Table 3.1, Figs. 3.9 and 3.10). Overall these results document the enhanced production efficiency of transgenic mice, and identify this factor as the key variable modulated to achieve faster growth at all ages.

3.8 DISCUSSION

PART 1 : *Interpretation of the Mass Budget*

3.8.1 *Regulation of Growth*:

Transgenic rat growth hormone mice clearly reveal the impact of exaggerated growth on the integration of other features (Tables 3.1, 3.2, and 3.3). Consistent with Kajiura and Rollo (1994), transgenic mice achieved much larger body sizes than normal mice (Fig. 3.1). Mean dry body mass of transgenic mice was 129% ($p < 0.0005$) and 148% ($p < 0.0005$) that of normal mice for early and late growth, respectively. Ultimately, transgenics achieved body sizes double that of normals (data not shown). Such gains were greater than those reported for other transgenic mice engineered with bovine, ovine, human, or porcine GH genes (Nagai *et al.* 1990; Pomp *et al.* 1992; Knapp *et al.* 1993). Rat GH possesses comparable structure and activity to that of endogenous mouse GH (Schindler *et al.* 1972), which may explain our stronger results. GH function varies among genes from other species, possibly due to differences associated with regulatory sequences in flanking regions of the trangenes, the number of transgenes, or their site of incorporation (Stefaneanu *et al.* 1993). For example, human GH mice display spontaneous lactation but mice with bovine GH transgenes do not (First and Haseltine 1991). Our study partially served as an independent replication of Kajiura and Rollo (1994), who examined only the late growth phase. The present results confirm that resource allocation has remained highly stable across several generations.

A difference in growth rates of younger normal or transgenic mice was not statistically

resolved even though transgenic mice were significantly larger by this time (129%, $p < 0.0005$, see Table 3.1 and Fig. 3.1). Body size is a cumulative process, and even small differences in exponential growth could lead to rapid divergence. Thus, the problem is undoubtedly an inability to discern the small differences in growth rates at younger ages, despite their visible phenotypic consequences. Other studies failed to detect any divergence in body sizes or growth rates between transgenic mice and controls less than 4 weeks old, and other authors suggest that mice may only respond to GH at later ages (Mathews *et al.* 1988; Bartke *et al.* 1991).

For both transgenic and normal mice, maximal growth was expressed during early ages and rate varied little between groups. A key insight is then, that transgenic mice never express a rate of growth that exceeds that achieved by normal mice at least sometime in their life (i.e. naturally evolved maximal growth rates are not exceeded). Nevertheless, transgenic mice were already doing things differently during the early growth period (i.e. reduced feeding and higher efficiencies, Figs. 3.4, 3.7, and 3.8), suggesting that rGH was already exerting strong impacts. Normal animals subsequently attenuated their growth rates faster, highlighting that improved growth performance of transgenic mice was obtained by delayed attenuation of otherwise normal growth capabilities. This implies that engineering elevations in growth capability beyond levels already expressed in normal animals might require redesign of other features limiting the system (e.g. duplications or upregulation of downstream elements of the growth control axis such as GH receptors or insulin-like growth factors). Such a model is consistent with the "principle of symmorphosis" derived from studies of respiratory physiology (Weibel *et al.* 1991; Rollo 1994). This principle recognizes that organisms evolve

to achieve the maximal outputs that normal experience requires but little more. If organisms are rarely "over built", then truly extraordinary responses may require redesign of numerous elements (i.e. genetic reorganization). These results have strong implications for genetic engineering. Shea *et al.* (1987) suggested that transgenic mice do not have extended growth compared to normal mice, but our results indicate that transgenic mice have slower attenuation of growth and males also continued growing into later ages (data not shown) (Fig. 3.1).

3.8.2 *Mechanisms of Growth Enhancement*:

Responses to artificial selection for simple weight gain in mice can result from numerous mechanisms. Animals may express either greater lean growth rates or they may repond via increased obesity. If larger lean sizes are obtained, they may result from either changes in growth rates, or changes in the targeted mature body size. These two aspects are under somewhat separate control, the body set point residing in the hypothalamus, while growth rate is mediated via the GH axis (Bernardis and Tannenbaum 1987; Bootland *et al.* 1991; Mosier *et al.* 1993). Changes in growth rates via the GH axis can result from modifications involving somatostatin, GH releasing hormone, GH binding protein, GH receptor (GHr), insulin-like growth factors (IGF-I and IGF-II), their binding proteins, or IGF receptors (see Thissen *et al.* 1994). It is not surprising, given this complexity, that not all changes in growth obtained via mutations or selection simply involve changes in levels of GH (Bootland *et al.* 1991). Nevertheless, this is one possible response. For example, in the artificially selected Goodale Giant mouse strain, GH levels are elevated and they express a

similar syndrome as seen in transgenic GH mice. This includes reduced reproduction, and decreased longevity (Logsdon and Nash 1977; Mukuni and Nash 1979). Similarly, large breeds of dogs are analogous to GH mice in having elevated levels of GH and IGF-I (Pendergrass *et al.* 1993). Our results support the dichotomy between growth rate control and targeted body size, since female Supermice were on average about 10-15 grams smaller than males, despite the fact that circulating levels of GH are probably saturated in both sexes. Furthermore, we recently crossed the transgenic rGH mouse with the hyperphagic "Tubby" strain. Preliminary results indicate no substantial improvement in maximal growth in hybrids but males and females now often grow to identical sizes (C. D. Rollo, unpublished data). It also seems significant that not only do these crosses show no evidence of the usual increase in size associated with hybridization, but the size of genetically engineered mice is remarkably similar to that obtained at selection limits following artificial selection.

Andersson *et al.* (1994) identified regions of the pig genome modified by artificial selection for enhanced growth and leaness in the large white boar. They examined genetic markers segregating in hybrids derived from this domesticated stock, and the ancestral European wild boar. A region influencing growth, fat deposition, and intestinal length was identified on chromosome 4, whereas the genes coding GH, GHr and IGF-I are located on chromosomes 12, 16, and 5, respectively. Our findings that transgenic mice express a very similar phenotype to other strains with highly exaggerated growth suggests some general implications, but clearly specific mechanisms may be relatively diverse.

3.8.3 *Feeding Regulation*:

It is commonly claimed that selection for faster growth or large size results in elevated feeding rates (Fowler 1962; McCarthy 1980; Roberts 1981; Malik 1984; Salmon *et al.* 1990). However, many of these studies refer to whole-organism intakes and it is hardly surprising that bigger animals eat more. On an age-related basis, older transgenic mice similarly consume greater absolute quantities of food in keeping with their larger size (Fig. 2), but when mass-specific rates were considered they actually ate less (Kajiura and Rollo 1994). Several studies documented depressions in mass-specific feeding in other transgenic and artificially selected rodent strains (Etherton *et al.* 1987; Campbell *et al.* 1989; Pursel *et al.* 1989, Steele and Pursel 1990; Pomp *et al.* 1992). Mass-specific feeding rates sometimes do repond to selection for larger sizes or growth rates (McCarthy 1980; McCarthy and Roberts 1989), but many other such experiments yield feeding rates that, like those reported here, are unchanged or actually decline in more rapidly growing animals (McPhee *et al.* 1980; Roberts 1981; Salmon *et al.* 1990). The present study clarifies that the reduced feeding observed in transgenic rGH mice is not simply an artifact associated with the allometric scaling of food consumption on body size (i.e. larger animals eat relatively less). Figure 3.3 clearly shows that for young, rapidly growing Supermice, those of equivalent size to normal controls ate lower absolute quantities of food, *even on a whole-organism basis*. Kajiura and Rollo (1994) proposed that transgenic GH, expressed mainly in the liver, may suppress appetite by its known negative feedback on production of endogenous GH in the pituitary (Sotelo *et al.* 1993). Although this could be a factor in transgenic GH animals, it does not explain the similar results obtained by artificial selection reviewed above.

A recent model suggests why transgenic mice may fail to adjust their feeding to regulate global energy demands. Webster (1993) proposed that feeding in rodents is regulated primarily to meet growth and protein accretion. Energy may only be regulated to the extent that it is needed to meet these contingencies. Thus, rats of various strains regulated protein accretion to a single precise level across diets varying widely in protein and energy content. Where excess energy was consumed in doing so, it was deposited as fat in a strain-specific, but otherwise unregulated manner (Webster 1993). Since we show that the main adjustment for varying growth rates is via altered production efficiencies rather than consumption, then improved efficiency to obtain greater growth could well result in lower specific feeding requirements. The impact of the abnormal energy demands of growth on other functions in transgenic mice would not be visible to the regulatory apparatus if it is only the need to meet growth demands that is monitored, and transgenic mice certainly achieve that objective well. Such a regulatory system could evolve if conditions of elevated growth are normally associated with conditions of abundant food energy and feeding regulation then shifts to emphasize the procurement of adequate protein, which overrides energetic considerations.

Very recently, a hormone signalling the size of fat reserves has been identified, and levels of this hormone are also associated with important shifts in metabolism, behaviour, and thermogenesis (Zhang *et al.* 1994; Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995). Indeed, the conflicting results on feeding regulation may arise because regulatory priorities shift under varying nutritional environments. Thus GH is associated with increased fat metabolism and reductions in fat reserves. The new obesity hormone (Leptin) is likely to interdigitate with the dynamic interplay between the GH and insulin axes.

Attempts to "improve" the growth, body composition, and feed efficiencies of agricultural species by insertion of GH genes have been marred by infertility, decreased longevity, reduced behavioural vigour, reduced feeding rates, and physiological ailments similar to the syndrome expressed in transgenic GH mice (Palmiter *et al.* 1982, 1983; Hammer *et al.* 1985*a,b*; Bartke *et al.* 1988; Doi *et al.* 1990; Brem *et al.* 1989; Rexroad *et al.* 1989; Pursel *et al.* 1989, 1990; Wall *et al.* 1990; Naar *et al.* 1991; Knapp *et al.* 1993; Wolf *et al.* 1993; Kajiura and Rollo 1994; Lachmansingh and Rollo 1994). The present results clarify and extend the insight of Kajiura and Rollo (1994), that such shortcomings result from a failure to increase resource intake to offset elevated costs of growth and consequent shortfalls in support of other functions (Fig. 3.4).

3.8.4 *Assimilation Efficiencies*:

Assimilation efficiencies of male transgenic mice, although slightly greater (a 3% difference) during the early mass budget ($p < 0.008$), were not statistically resolved from normals during later growth (Table 3.1). McCarthy (1980) noted that assimilation efficiency rarely responds to artificial selection for growth in mice. Our results reinforce that AE is not an important aspect in enhanced growth performance in transgenic mice and shows little variation with ontogeny.

3.8.5 *Respiration*:

Respiration rates were significantly lower in transgenic mice compared to normals (Table 3.1 and Figs. 3.5 and 3.6). The fact that transgenic mice had higher growth rates for

a given level of respiration may reflect the diversion of energy toward growth at the expense of behavioural, reproductive, and longevity assurance functions (see below). Respiration rates, however, were determined using gains in body mass as the practical index of growth. Millward *et al.* (1976) cautioned, however, that increases in body mass represent only a small portion of the total energy required to produce them and that mass budgets do not allow the factoring in of the costs of growth. We are currently comparing the oxygen consumption rates of transgenic and normal mice to provide a more accurate measure of the energetic costs of growth. Respiration rates at younger ages were higher than in older mice, as expected, but the relative difference between normal and transgenic animals remained relatively constant with age. Although older transgenic mice and normals showed a relatively singular relationship between consumption rates and respiration rates, the younger transgenic mice had lower consumption rates for any given level of respiration (Fig. 3.5), a situation that is consistent with our interpretation of transgenic mice being energetically constrained.

3.8.6 *Production Efficiencies*:

Mice selected for larger body sizes or rapid growth, universally show greater production efficiencies compared to unselected controls. Enhanced production efficiencies, reduced behavioural activity, decreased longevity, and infertility, emerge in animals artificially selected for large body mass, those injected with growth hormone, and in transgenic GH animals (MacArthur 1949; Falconer 1953; Timon *et al.* 1970; Sutherland 1974; Eklund and Bradford 1977; McCarthy 1980; Roberts *et al.* 1980; Malik 1984; Bishop and Hill 1985; Calvert *et al.* 1986; Bernier *et al.* 1986; Grosbeck *et al.* 1987; McCarthy and Roberts 1989;

Pursel *et al.* 1989; Salmon *et al.* 1990; Bootland *et al.* 1991; Wolf *et al.* 1991; Pomp *et al.* 1992). Barnard *et al.* (1983), Bronson (1988), and Moruppa (1990) previously concluded that reduced behavioural activity could enhance growth efficiency, a result we confirmed in transgenic mice (Lachmansingh and Rollo 1994). Alternatively, selection for small body size yields reduced growth efficiency and hyperactivity. Dietary restriction induces a physiological reorganization that includes reduced growth rates, and body sizes. This is also associated with reduced production efficiencies as mass-specific feeding rates are held relatively constant while growth rates are reduced (see Ross *et al.* 1985). Hyperactive "spinner" mice have significantly reduced growth, possibly as a direct trade-off of behavioural activity with growth efficiency (Lachmansingh and Rollo 1994). Our results indicate that the ontogeny of growth efficiency is also consistent with a very close linkage between efficiencies and growth rates, with higher production efficiencies being associated with higher growth rates (see also Malik 1984). Transgenic rGH mice achieved enhanced growth via improvements in gross and net production efficiencies and maintenance of relatively greater efficiency into later ages (i.e. an ontogenic extension) (Table 3.1, and Figs. 3.7 and 3.8, Figs. 3.9 and 3.10). Since the improved efficiencies were likely obtained via trade-offs and detrimental impacts on other features, this is probably better interpreted as a physiological "stress" rather than as an improvement in ecologically relevant design. The fact that normal animals showed positive growth across the experiment (Fig. 3.1), but feeding plateaued at about 30 d of age (Fig. 3.2), suggests that growth efficiency must be the dominant adjustment following this age. It might be very worthwhile to examine the scaling of the digestive system with age, since the feeding plateau suggests either a homeostatic set point (perhaps metabolic) or a constraint on further

digestive processing.

PART 2: ***Synthesis of the Transgenic rGH Mouse Results with Other Important Areas***

3.8.7 ***The Growth : Longevity Axis , Modulation of the Mouse Adaptive Suite***:

A key focus of gerontology has been the enhanced longevity obtained in rodents on calorically restricted diets (Weindruch and Walford 1988). This likely reflects an evolved reaction norm that adaptively adjusts physiological and life-history features to resource supply (Totter 1985; Holliday 1989; Graves 1993; Rollo 1994). Thus, within the normal limits of size for *Mus musculus*, larger body mass or selection for rapid growth yields earlier maturation, larger litters, and faster litter production (Falconer 1953; Roberts 1961; Bradford *et al.* 1980; Bronson 1984), characteristics suited for maximizing contributions to rapidly growing populations in r-selective habitats. The dietary restriction norm also modulates reproductive rates with those fed *ad libitum* being larger, faster-growing animals with greater reproductive effort, and shorter lives. Smaller, slower-growing mice may have greater fitness under restricted food regimes because they can potentially wait out the shortage , spread out reduced reproductive efforts across a greater period of reproductive competence, or even obtain greater lifetime fecundity (see Roberts 1961).Such characteristics would maximize fitness in high density populations, or other resource limiting environments where populations are not growing rapidly.

Dietary restriction induces decreased body size (Conn *et al.* 1993), reduced levels of circulating GH and IGF (Breese *et al.* 1991; Thissen *et al.* 1994), thermogenesis that is reduced or has increased circadian amplitude (Forsum *et al.* 1981; Cheney *et al.* 1983;

McCarter *et al.* 1985), and delayed, reduced, or suppressed reproduction (Holehan and Merry 1986). Concomittently, immunological functions, longevity, behavioural vigour, and reproductive competence are enhanced and extended into older ages (Goodrick *et al.* 1982; Igram and Reynolds 1987; Weindruch and Walford 1988; Holliday 1989; Mournier *et al.* 1989; Finch 1990; Means *et al.* 1993; Graves 1993). The regulated reallocation of energy evident under dietary restriction can be rationalized with Webster's (1993) model of feeding regulation if global energy regulation is emphasized when there are insufficient resources to attain targeted growth rates. In normal circumstances, diets that support rapid growth are unlikely to be deficient in energy, and so growth could well be utilized as a reliable signal for the prevailing energy supply as well.

Regulation of the dietary restriction norm is undoubtedly linked to elements of the growth regulating axis. The idea was rejected earlier by Ingram and Reynolds (1987), because GH injections failed to induce growth in restricted rodents, but more recent findings confirm precise nutritional modulation of the growth regulating axis and suggest that the major mechanism may involve changes in GH receptor densities or other factors leading to lower levels of circulating IGF, rather than circulating levels of GH (Breese *et al.* 1991; Straus and Takemoto 1991; Thissen *et al.* 1994). Failure to respond to GH in restricted rodents, is more likely due to regulated GH resistance (Thissen *et al.* 1994). It seems highly significant that levels of IGF-I are strongly associated with dietary protein levels (Thissen *et al.* 1994), and the same signal is implicated in feeding regulation (Webster 1993). A role for growth as a key factor in the dietary restriction response has also been questioned because restriction of mature animals may also obtain lifespan extensions (Yu 1987). This interpretation ignores

the fact that such restricted animals usually lose weight, the yields in increased longevity are relatively minor, and stationary growth (i.e. continued protein and cell turnover with no further increase in size) continues in such animals.

The main response to caloric restriction is via reduced growth rates and smaller adult sizes, while holding mass-specific feeding and metabolic rates constant (McCarter *et al.* 1985; Yu *et al.* 1985; Duffy *et al.* 1989; Masoro 1993). Such regulatory organization could also explain the failure of transgenic mice to increase their specific feeding rates in response to growth that is induced by genes outside of the regulatory organization. Transgenic mice and normal mice show similar compensatory feeding following short periods of starvation (about 1.3 to 2.0 times normal intakes), suggesting that there is residual capacity in the digestive system at least for short-term adjustments (L. N. DelCotto and C. D. Rollo, unpublished data). Interspecifically, however, compensatory scope is restricted to elevations of about 2 to 6 times normal, probably due to digestive or respiratory constraints (reviewed by Rollo 1994). In pigs, it is suggestive that selection for enhanced growth increases the length of the small intestine (Andersson *et al.* 1994). The degree to which such changes represent phenotypic plasticity as opposed to genetic changes remains unknown.

The idea that support of the soma might be reduced under conditions favouring high productivity and rapid population growth is predicted by the disposable soma theory of aging (Kirkwood 1977; Holliday 1989; Heydari and Richardson 1992; Masoro 1993). Our transgenic mice live about half as long as normal (Lachmansingh and Rollo 1994), and other transgenic mice also show characteristics consistent with accelerated aging (Bartke *et al.* 1989, 1991; Brem *et al.* 1989; Pendergrass *et al.* 1993; Sotelo *et al.* 1993; Steger *et al.* 1993;

Wolf *et al.* 1993). Thus, the regulatory organization that yields extended lives in slow-growing rodents might also be responsible for regulated reductions in somatic support in those with abnormally exaggerated growth.

Although there are numerous mechanisms under investigation, the best evidence suggests that dietary restriction modulates aging rate by upregulating investments in defense, repair, or replacement in response to damage inflicted by free oxygen radicals (see Harman 1971, 1992; Rao *et al.* 1990; Bernstein and Bernstein 1991; Pacifici and Davies 1991; Cutler 1992; Heydari and Richardson 1992; Orr and Sohal 1994, Rollo 1994). Thus, accelerated aging might arise either via regulated allocation to "longevity assurance" or if high energetic investments in growth directly compromise investments in longevity assurance systems.

Papers in preparation (Rollo *et al.*, unpublished) show that providing carbohydrate supplements alleviates behavioural lethargy of male transgenic mice, yields enhanced longevity (at least in female transgenic mice), and improves female reproduction. We found that transgenic mice display higher age-specific levels of superoxide radical (a key free radical) and lipid peroxidation (a key measure of aging rates and free radical damage) (papers in preparation). These results support our theory that longevity assurance investments are reduced in transgenic mice due to their naturally evolved regulatory organization, and/or direct compromise of resource allocation under constant energy supplies.

The norm of reaction associated with dietary restriction constitutes a crucial axis modulating resource allocation tactics in response to environmental quality. The maladaptive syndrome expressed by transgenic mice is consistent with superimposing unregulated growth onto an organization otherwise evolved to adjust body sizes, reproduction, and longevity

assurance investments while holding gram-specific rates of metabolism and nutrient processing constant. Changes in production efficiency emerge as the key factor impacted by all mechanisms modifying body sizes and growth rates, including dietary restriction, artificial selection, mutations affecting body size, and transgenic GH mice.

3.8.8 *Allometry: Implications for an Ultimate Synthesis*:

Larger phenotypes can be achieved by various combinations of increased growth rates or extended durations of growth. The dream of achieving giant, normally functioning animals by the simple insertion of GH genes may be naive. Such manipulations appear to impact instead on intraspecific regulatory structure that rather ubiquitously functions to increase reproductive effort while reducing somatic support in larger phenotypes (i.e. dietary restriction responses are similar across diverse phylogenies, Weindruch and Walford 1988). Intraspecific selection for larger size generally yields faster-growing animals. Interspecifically, larger sizes and greater longevities are positively correlated but this appears to involve longer durations of slower growth, reduced reproductive efforts, and specific upregulation of longevity assurance investments to offset metabolically-induced damage (see Cutler 1992). Thus, selection for increased body size within species tends to accelerate rates, but selection across species appears to involve rate reductions. Such a situation could go a long way towards explaining differences in intraspecific allometry versus interspecific allometry as well as the relativistic telescoping of life-history features across species that yields numerous life-history invariants (Peters 1983; Calder 1984; Schmidt-Nielsen 1984; Reiss 1989; Millar and Hickling 1991; Charnov 1993). That is, larger animals within species may live at faster rates,

but as species-specific body size increases, physiological rates decline and the rate of living slows down. The route to interspecific evolution of larger species is unlikely to be via selection for faster intraspecific growth rates.

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TABLE 3.1. Comparison of means and SE for various measures of the dry mass budget of transgenic Supermice and normal male *Mus musculus* aged 25 to 40 d and 47 to 62 d old.

	EARLY GROWTH (DAY 25 TO 40)						LATE GROWTH (DAY 47 TO 62)					
	NORMAL		TRANSGENIC				NORMAL		TRANSGENIC			
MEASUREMENT	MEAN	SE	MEAN	SE	*t*-test	% (T/N)	MEAN	SE	MEAN	SE	*t*-test	% (T/N)
Body Mass (g)	4.52	0.09	5.81	0.17	$p < 0.0005$	129	7.14	0.08	10.58	0.17	$p < 0.0005$	148
Growth Rate (mg/mg dry body mass per day)	0.044	0.002	0.045	0.002	ns	102	0.006	0.0003	0.011	0.0005	$p < 0.0005$	183
Consumption Rate (mg/mg dry body mass per day)	0.868	0.010	0.746	0.010	$p < 0.0005$	86	0.612	0.011	0.563	0.005	$p < 0.0002$	92
Faecal Rate (mg/mg dry body mass per day)	0.301	0.005	0.246	0.004	$p < 0.0005$	82	0.204	0.004	0.193	0.002	$p < 0.01$	95
Assimilation Rate (mg/mg dry body mass per day)	0.567	0.007	0.500	0.009	$p < 0.0005$	88	0.406	0.009	0.371	0.005	$p < 0.001$	91
Respiration Rate (mg/mg dry body mass per day)	0.524	0.007	0.455	0.008	$p < 0.0005$	87	0.400	0.009	0.360	0.005	$p < 0.0003$	90
Assimilation Efficiency (%)	65.32	0.39	66.98	0.46	$p < 0.008$	103	66.31	0.46	65.60	0.54	ns	99
Gross Production Efficiency (%)	5.02	0.19	6.00	0.16	$p < 0.0003$	120	1.00	0.06	1.93	0.08	$p < 0.0005$	193
Net Production Efficiency (%)	7.70	0.31	8.96	0.23	$p < 0.002$	116	1.51	0.09	2.94	0.12	$p < 0.0005$	195

NOTE: Probabilities were determined by *t*-tests. Each group was comprised of 20 mice. Columns labeled % (T/N) express results for the transgenic mice as a percentage of the normals. All variables except for body mass and efficiency are indicated in mass-specific units.

TABLE 3.2 Pairwise comparisons of *early growth* correlation coefficients between various dry mass budget measurements for transgenic Supermice (left side of the diagonal) and normal male *Mus musculus* (right side of the diagonal).

	NORMAL								
TRANSGENIC	1	2	3	4	5	6	7	8	9
1. Dry Body Mass	...	**-0.70**	-0.19	-0.36	-0.01	0.15	0.34	**-0.61**	**-0.64**
2. Growth Rate	**-0.60**	...	0.04	0.23	-0.11	-0.33	-0.30	**0.95**	**0.96**
3. Consumption Rate	-0.39	**0.81**	...	**0.77**	**0.88**	**0.83**	-0.17	-0.27	-0.22
4. Faecal Rate	0.02	0.36	**0.47**	...	0.37	0.30	**-0.76**	-0.03	0.10
5. Assimilation Rate	**-0.45**	**0.77**	**0.93**	0.11	...	**0.98**	0.32	-0.37	-0.40
6. Respiration Rate	-0.39	**0.67**	**0.90**	0.05	**0.99**	...	0.37	**-0.56**	**-0.59**
7. Assimilaton Efficiency	-0.38	0.35	0.38	**-0.63**	**0.70**	**0.74**	...	-0.21	-0.37
8. Gross Production Efficiency	**-0.63**	**0.95**	**0.59**	0.25	**0.55**	0.44	0.26	...	**0.99**
9. Net Production Efficiency	**-0.55**	**0.88**	**0.50**	**0.44**	0.37	0.25	-0.01	**0.96**	...

NOTE: Underlined correlation coefficients indicate reversals in sign (+ or -) in comparison to corresponding coefficients for late growth. Significant correlation coefficients are indicated by bold font.

TABLE 3.3 Pairwise comparisons of *late growth* correlation coefficients between various dry mass budget measurements for transgenic Supermice (left side of the diagonal) and normal male *Mus musculus* (right side of the diagonal).

	NORMAL								
TRANSGENIC	1	2	3	4	5	6	7	8	9
1. Dry Body Mass	...	0.17	-0.30	**-0.52**	-0.21	-0.21	0.10	0.25	0.24
2. Growth Rate	-0.40	...	-0.25	-0.19	-0.22	-0.26	-0.01	**0.96**	**0.95**
3. Consumption Rate	**0.54**	0.21	...	**0.68**	**0.94**	**0.95**	0.28	**-0.49**	**-0.51**
4. Faecal Rate	0.11	-0.05	0.43	...	0.44	0.44	-0.41	-0.35	-0.31
5. Assimilation Rate	**-0.64**	0.24	**0.85**	-0.07	...	**0.99**	**0.58**	-0.44	**-0.49**
6. Respiration Rate	**-0.61**	0.15	**0.84**	-0.07	**0.99**	...	**0.58**	**-0.47**	**-0.52**
7. Assimilaton Efficiency	**-0.46**	0.20	0.27	**-0.55**	**0.68**	**0.67**	...	-0.09	-0.17
8. Gross Production Efficiency	-0.29	**0.98**	0.02	-0.13	0.08	-0.02	0.15	...	**0.99**
9. Net Production Efficiency	-0.21	**0.95**	-0.05	-0.02	-0.06	-0.16	-0.01	**0.98**	...

NOTE: Underlined correlation coefficients indicate reversals in sign (+ or -) in comparison to corresponding coefficients for early growth.Significant correlations are indicated by **bold** font.

FIG. 3.1 Relationship between age (*AGE* = log day) and mean dry body mass (*BODY MASS* = log grams) of normal (■) and transgenic (□) male *Mus musculus* for early and late growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: *BODY* MASS = 1.559(*AGE*) - 1.698, $r^2 = 0.970$, df = 14, $p < 0.0005$; **Transgenic**: *BODY MASS* = 1.508(*AGE*) - 1.511, $r^2 = 0.984$, df = 14, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal**: *BODY MASS* = 0.326(*AGE*) + 0.289, $r^2 = 0.991$, df = 14, $p <$ 0.0005; **Transgenic**: *BODY MASS* = 0.596(*AGE*) - 0.009, $r^2 = 0.995$, df = 14, $p < 0.0005$.

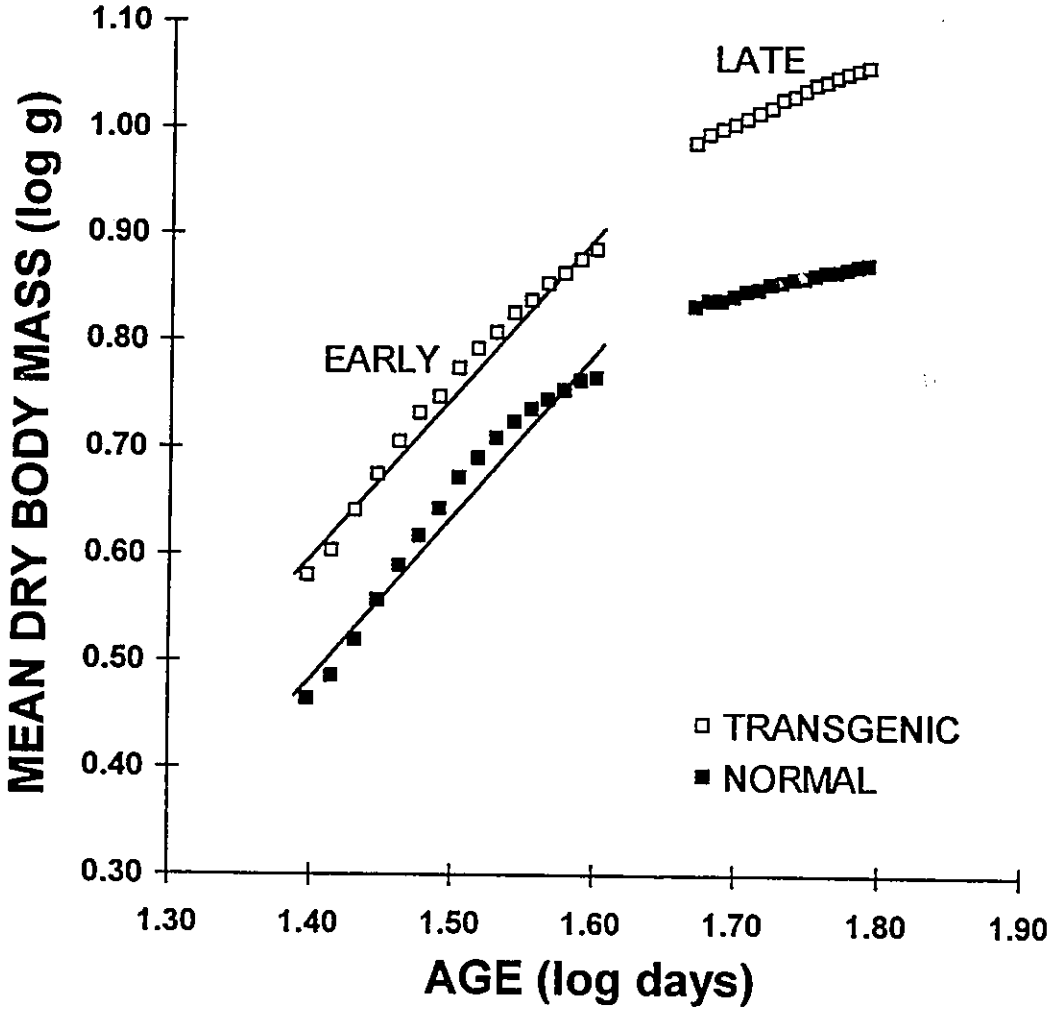

FIG. 3.1 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.2 Relationship between age (*AGE*) and age-related consumption rate (*ARCR*) of male *Mus musculus* for each of the 15 d of the early (normal ■ , transgenic □), and late (normal ●, transgenic ○) growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $ARCR = 123.440(AGE) - 31.568$, $r^2 = 0.718$, df = 13, $p < 0.0005$; **Transgenic**: $ARCR = 131.145(AGE) + 128.154$, $r^2 = 0.767$, df = 13, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal**: $ARCR = 9.669(AGE) + 3840.212$, $r^2 = 0.154$, df = 13, ns; **Transgenic**: $ARCR = 47.379(AGE) + 3390.018$, $r^2 = 0.573$, df = 13, $p < 0.001$.

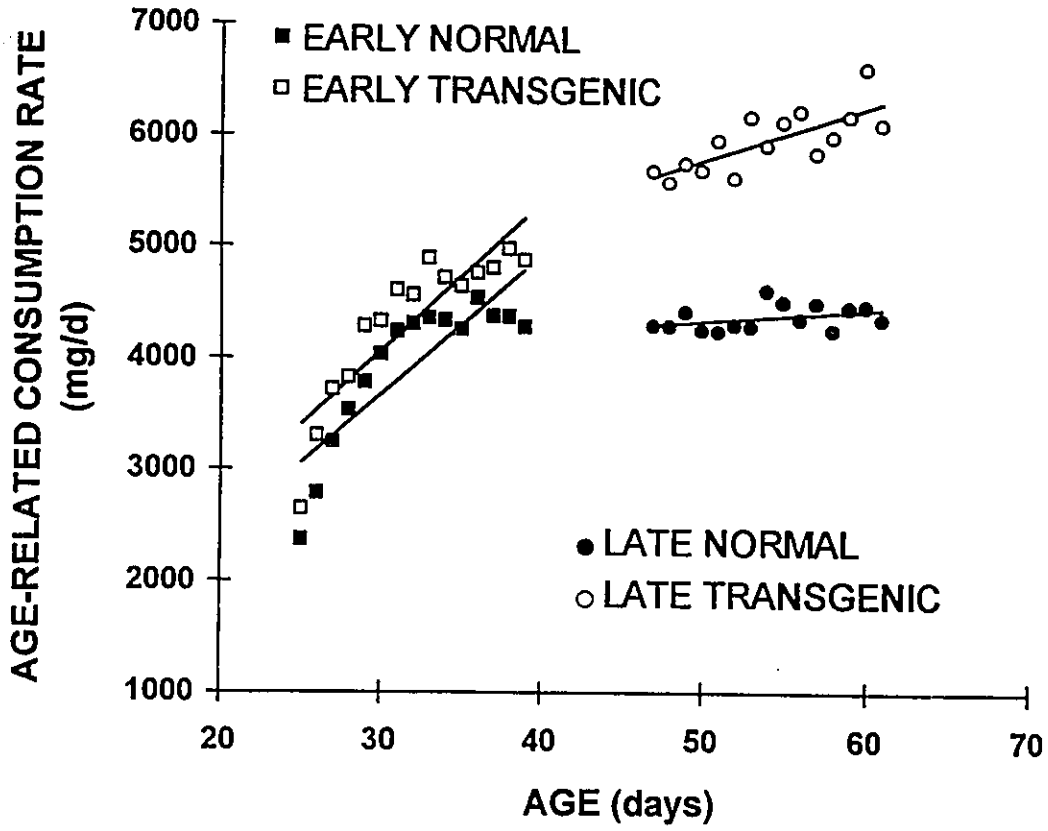

FIG. 3.2 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.3 Relationship between overall dry body mass (*BM*) and whole-animal consumption (*WACR*) of male *Mus musculus* for early (normal ■ , transgenic □) and late (normal ● , transgenic ○) growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $WACR = 0.789(BM) + 354.402$, $r^2 = 0.735$, df = 18, $p < 0.0005$; **Transgenic**: $WACR = 0.608(BM) + 794.094$, $r^2 = 0.809$, df = 18, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal** : $WACR = 0.324(BM) + 2046.708$, $r^2 = 0.103$, df = 18, ns; **Transgenic**: $WACR = 0.409(BM) + 1616.216$, $r^2 = 0.745$, df = 18, $p < 0.0005$.

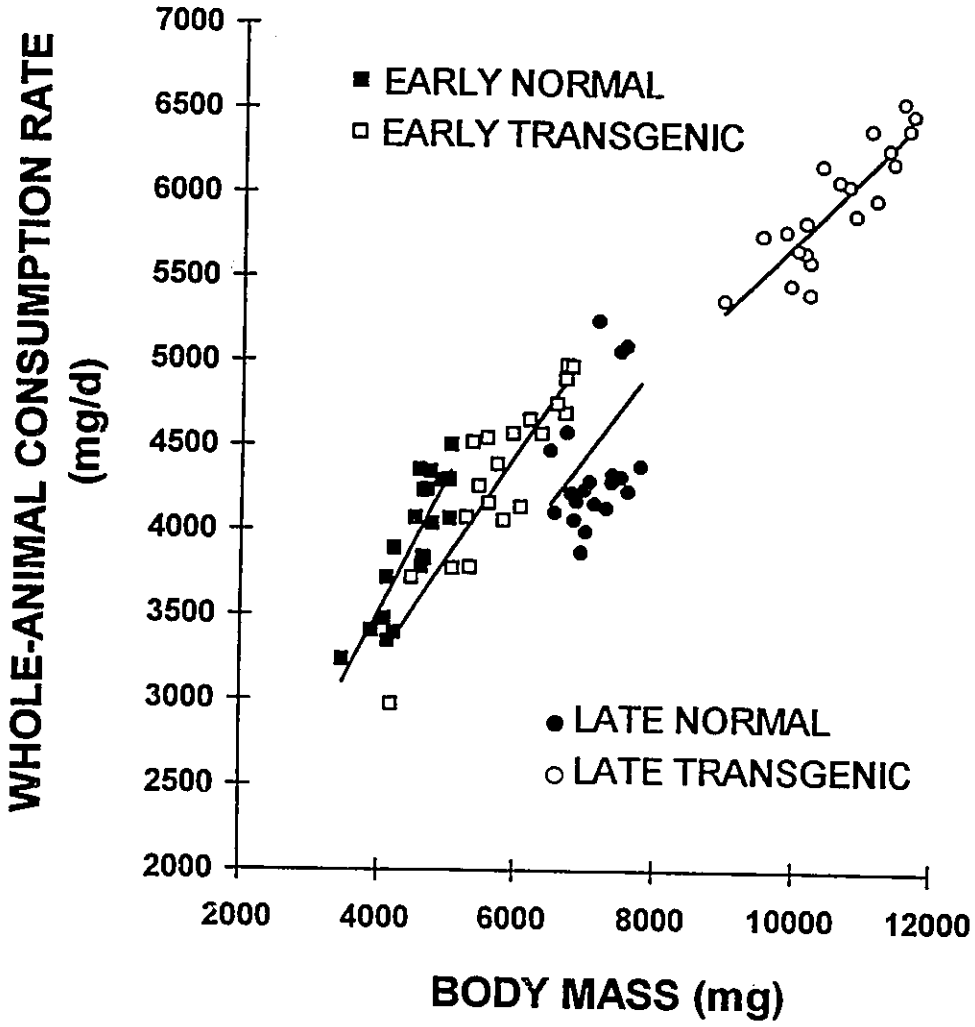

FIG. 3.3 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.4 Relationship between overall mass-specific rates of growth (*GR*) and consumption (*CR*) of normal (■) and transgenic (□) male *Mus musculus* for early and late growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $CR = 0.252(GR) + 0.857$, $r^2 = 0.002$, df = 18, ns; **Transgenic**: $CR = 5.043(GR) + 0.522$, $r^2 = 0.647$, df = 18, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal**: $CR = -8.412(GR) + 0.663$, $r^2 = 0.066$, df = 18, ns; **Transgenic**: $CR = 2.100(GR) + 0.540$, $r^2 = 0.046$, df = 18, ns.

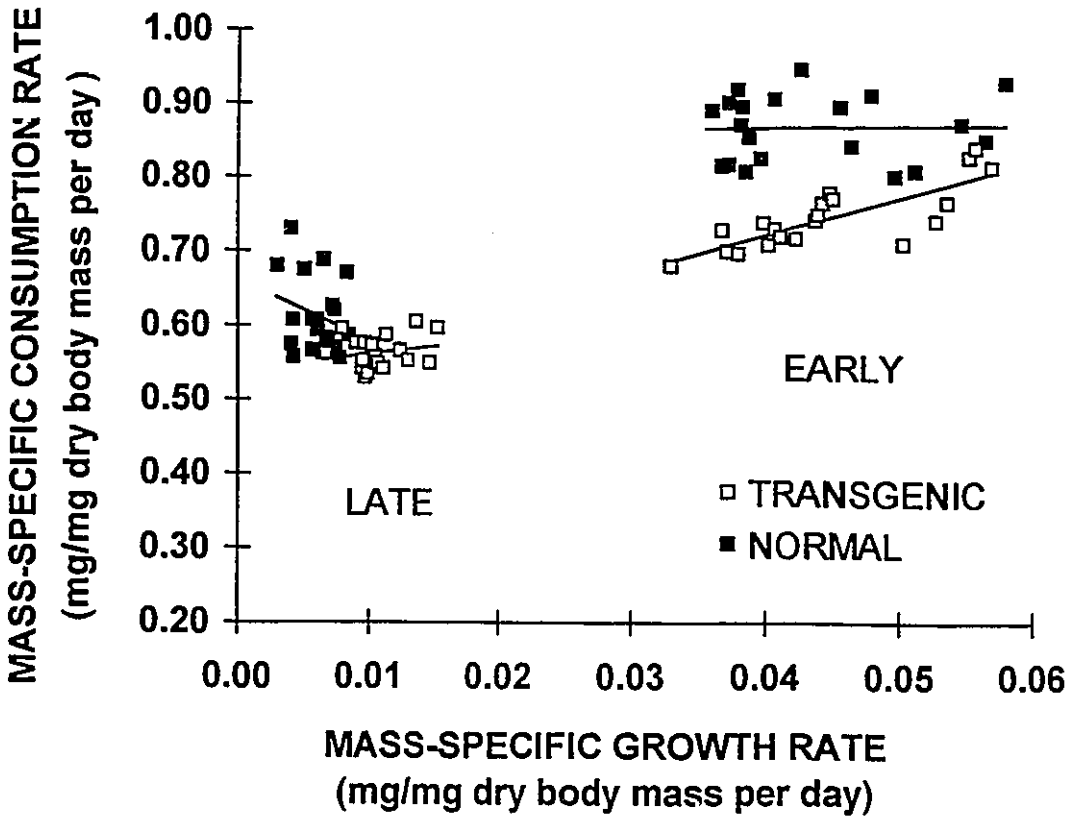

FIG. 3.4 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.5 Relationship between overall mass-specific consumption rate (*CR*) and respiration rate (*RR*) of male *Mus musculus* for early (normal ■, transgenic □) and late (normal ●, transgenic ○) growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $RR = 0.593(CR) + 0.008$, $r^2 = 0.681$, df = 18, $p < 0.0005$; **Transgenic**: $RR = 0.689(CR) - 0.059$, $r^2 = 0.803$, df = 18, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal**: $RR = 0.754(CR) - 0.062$, $r^2 = 0.893$, df = 18, $p < 0.0005$; **Transgenic**: $RR = 0.847(CR) - 0.117$, $r^2 = 0.706$, df = 18, $p < 0.0005$.

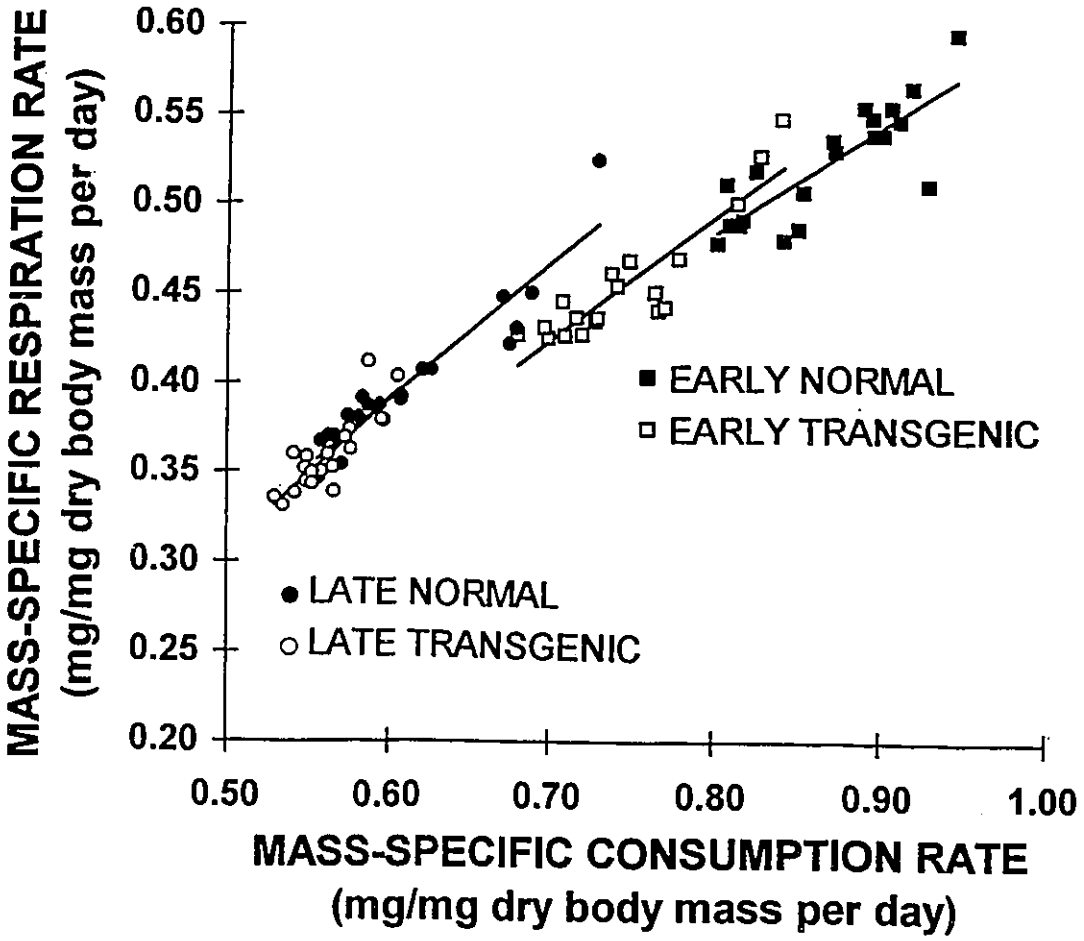

FIG. 3.5 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.6 Relationship between overall mass-specific growth rate (*GR*) and respiration rate (*RR*) of normal (■) and transgenic (□) male *Mus musculus* for early and late growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $RR = -1.495(GR) + 0.589$, $r^2 = 0.107$, df = 18, ns; **Transgenic**: $RR = 3.202(GR) + 0.311$, $r^2 = 0.453$, df = 18, $p < 0.001$. ***Late***: equations for the regression lines are as follows: **Normal**: $RR = -6.737(GR) + 0.441$, $r^2 = 0.066$, df = 18, ns; **Transgenic**: $RR = 1.448(GR) + 0.344$, $r^2 = 0.021$, df = 18, ns.

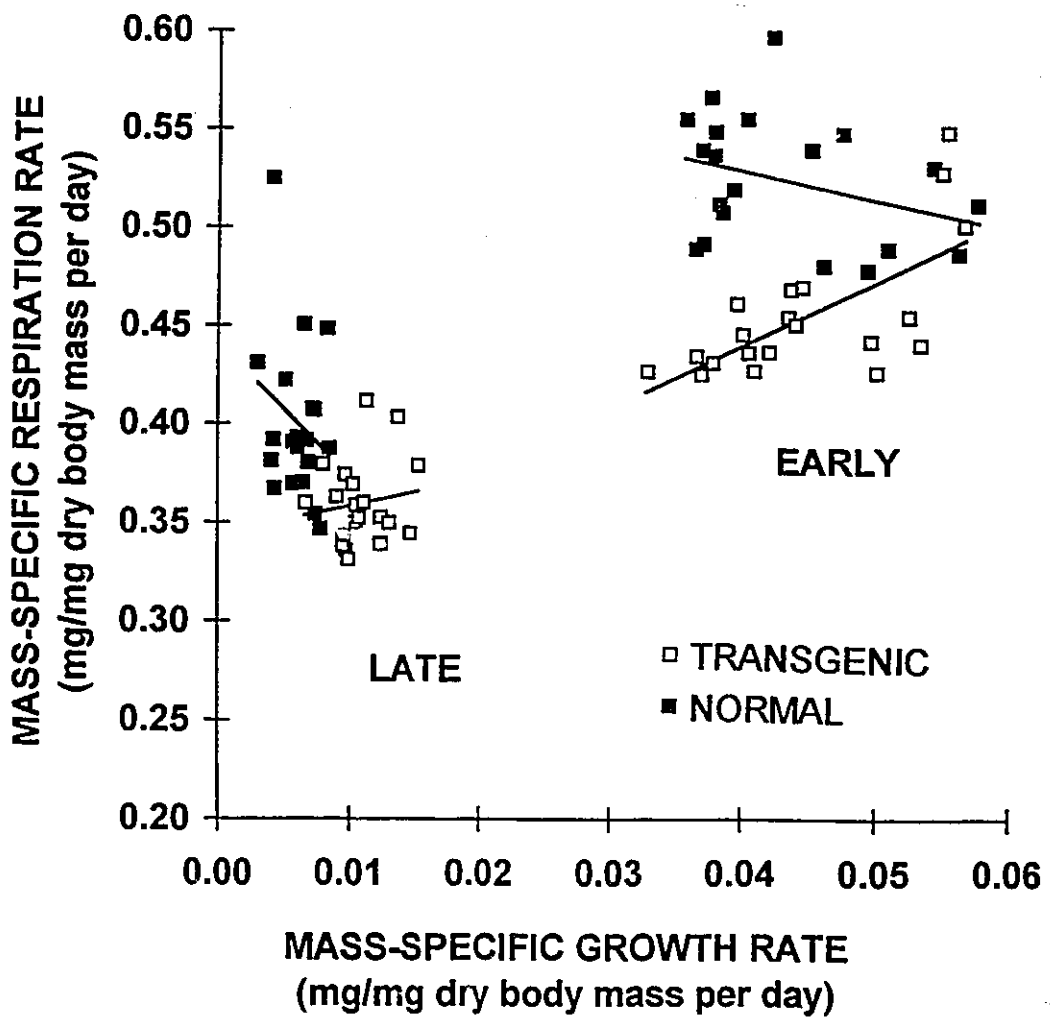

FIG. 3.6 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.7 Relationship between overall mass-specific growth rate (*GR*) and gross production efficiency (*GPE*) of normal (■) and transgenic (□) male *Mus musculus* for early and late growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $GPE = 113.607(GR) + 0.079$, $r^2 = 0.901$, df = 18, $p < 0.0005$; **Transgenic**: $GPE = 93.075(GR) + 1.187$, $r^2 = 0.897$, df = 18, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal**: $GPE = 172.513(GR) - 0.045$, $r^2 = 0.931$, df = 18, $p < 0.0005$; **Transgenic**: $GPE = 167.853(GR) + 0.108$, $r^2 = 0.960$, df = 18, $p < 0.0005$.

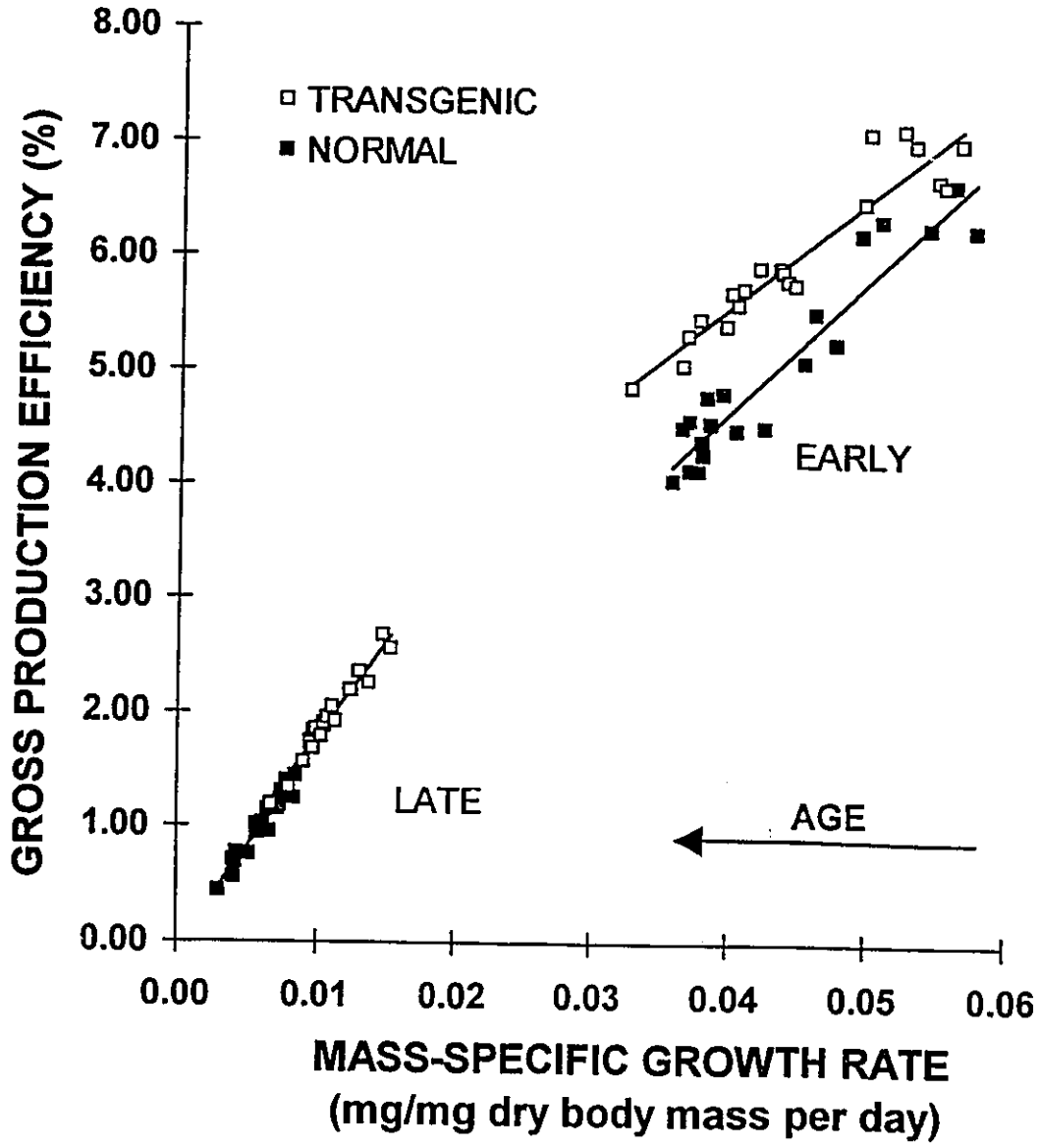

FIG. 3.7 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.8 Relationship between overall mass-specific growth rate (*GR*) and net production efficiency (*NPE*) of normal (■) and transgenic (□) male *Mus musculus* for early and late growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $NPE = 184.632(GR) - 0.333$, $r^2 = 0.912$, df = 18, $p < 0.0005$; **Transgenic**: $NPE = 114.924(GR) + 3.743$, $r^2 = 0.654$, df = 18, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal**: $NPE = 260.441(GR) - 0.067$, $r^2 = 0.907$, df = 18, $p < 0.0005$; **Transgenic**: $NPE = 256.549(GR) + 0.132$, $r^2 = 0.911$, df = 18, $p < 0.0005$.

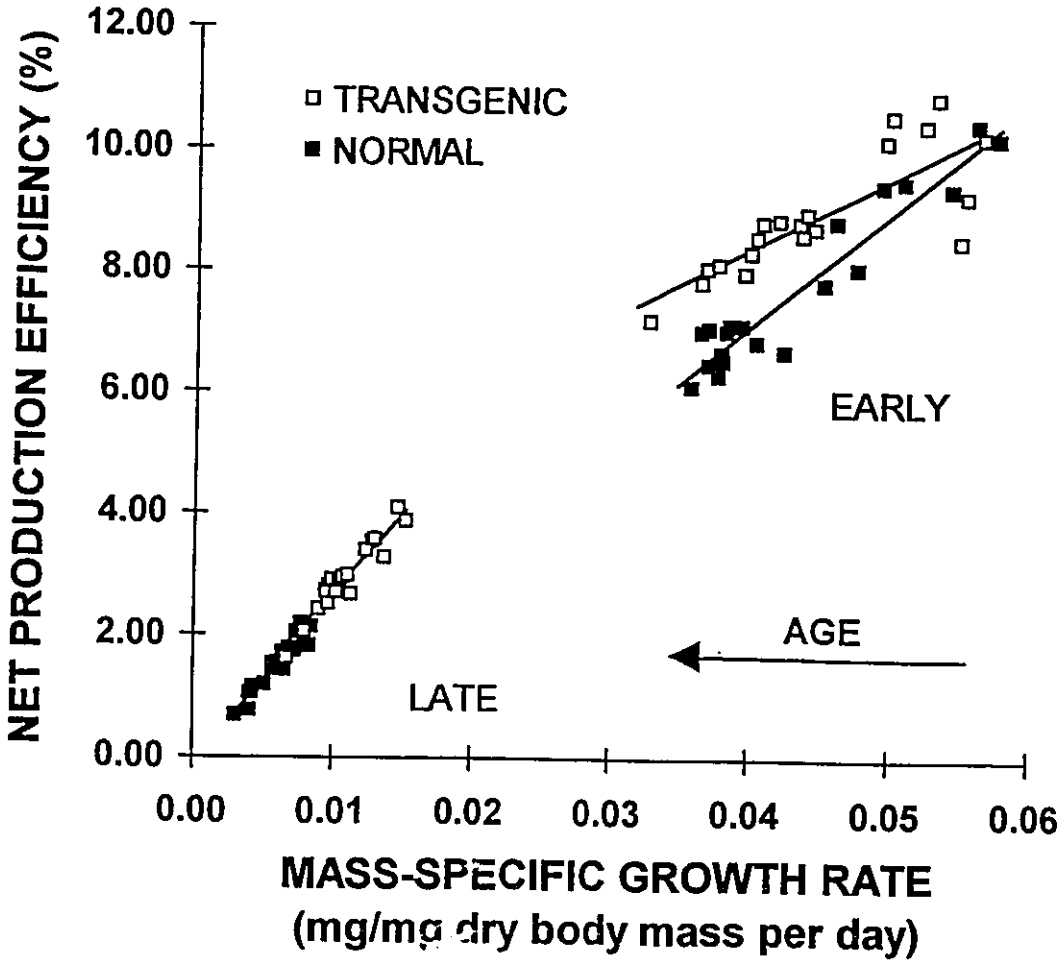

FIG. 3.8 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.9 Relationship between age (*AGE*) and gross production efficiency (*GPE*) of male *Mus musculus* for each of the 15 d of the early (normal ■ , transgenic □), and late (normal ●, transgenic ○) growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $GPE = -0.471(AGE) + 20.241$, $r^2 = 0.796$, df = 13, $p < 0.0005$; **Transgenic**: $GPE = -0.441(AGE) + 20.326$, $r^2 = 0.721$, df = 13, $p < 0.0005$. ***Late***: equations for the regression lines are as follows: **Normal**: $GPE = -0.054(AGE) + 3.868$, $r^2 = 0.184$, df = 13, ns; **Transgenic**: $GPE = -0.091(AGE) + 6.864$, $r^2 = 0.293$, df = 13, $p < 0.03$.

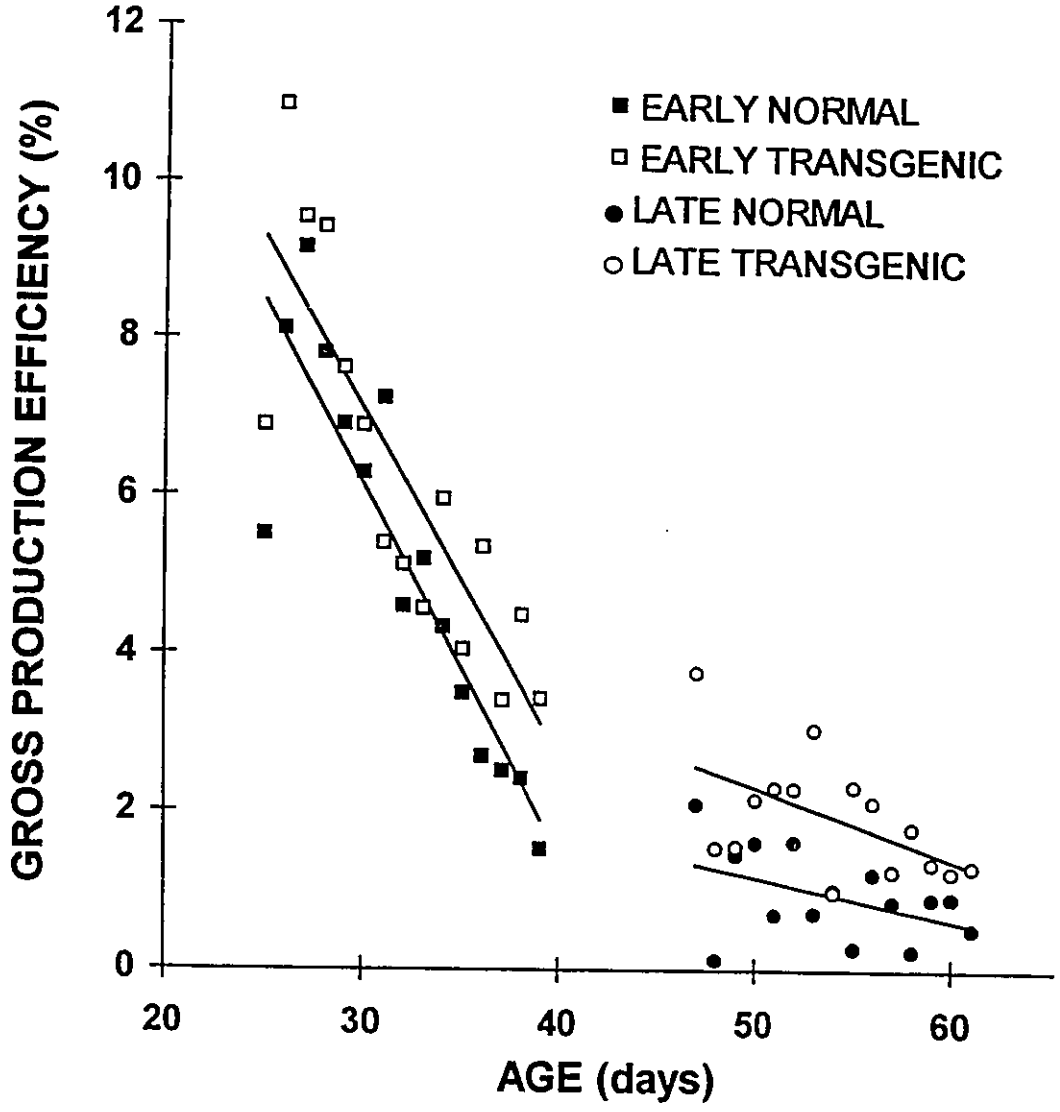

FIG. 3.9 KAJIURA, L. J., and ROLLO, C. D. 1995.

FIG. 3.10 Relationship between age (*AGE*) and net production efficiency (*NPE*) of male *Mus musculus* for each of the 15 d of the early (normal ■ , transgenic □), and late (normal ●, transgenic ○) growth phases. ***Early***: equations for the regression lines are as follows: **Normal**: $NPE = -0.691(AGE) + 30.009$, $r^2 = 0.797$, df = 13, $p < 0.0005$; **Transgenic**: $NPE = -0.680(AGE) + 31.065$, $r^2 = 0.664$, df = 13, $p < 0.0002$. ***Late***: equations for the regression lines are as follows: **Normal**: $NPE = -0.076(AGE) + 5.558$, $r^2 = 0.156$, df = 13, ns; **Transgenic**: $NPE = -0.111(AGE) + 8.851$, $r^2 = 0.210$, df = 13, ns.

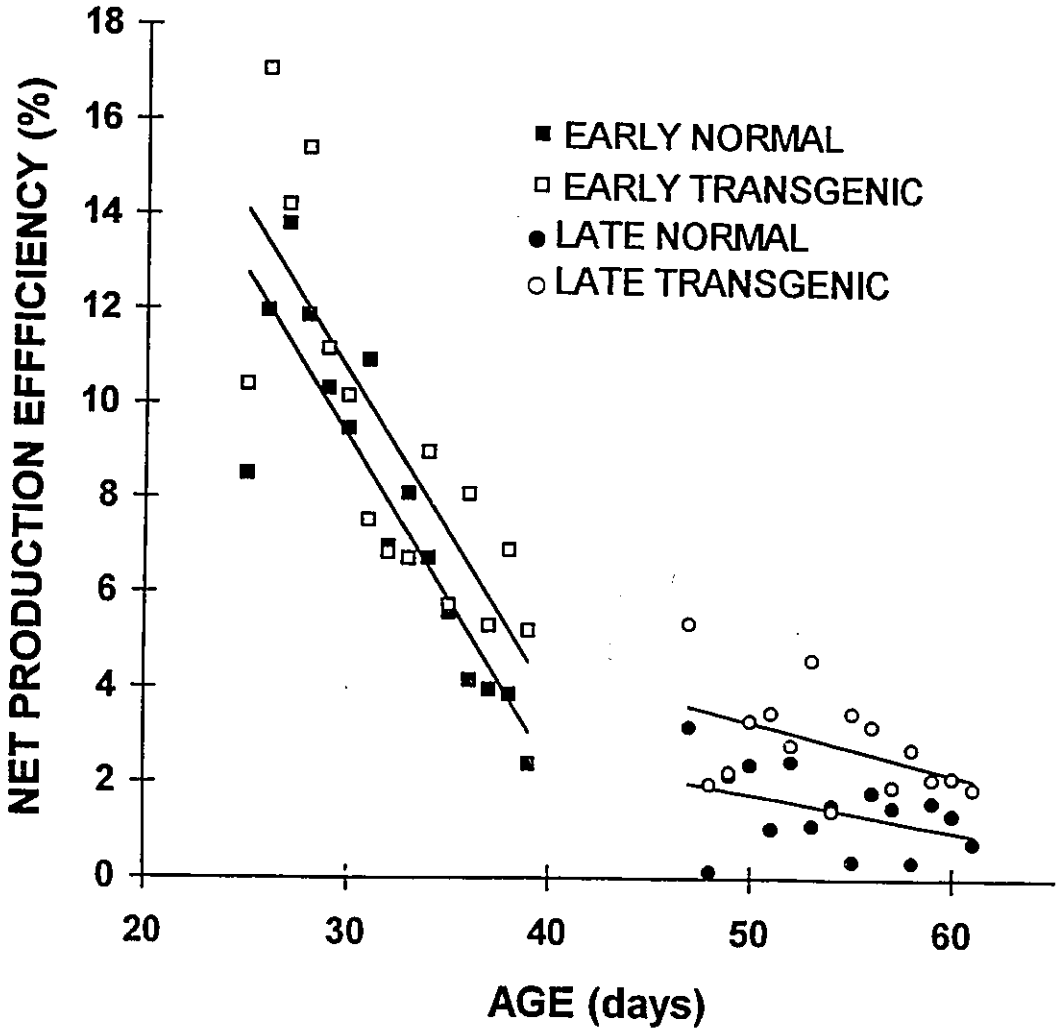

FIG. 3.10 KAJIURA, L. J., and ROLLO, C. D. 1995.

SECTION IV

CONCLUDING DISCUSSION AND GENERAL SUMMARY

4

CONCLUDING DISCUSSION AND GENERAL SUMMARY

In the present thesis, the impact of genetically engineered growth enhancement on mammalian physiological resource allocation was evaluated through comparisons of Supermice and their non-transgenic relatives. Dry mass budgets revealed three major insights. Firstly and perhaps most significantly, was the discovery that Supermice failed to adjust their mass-specific feeding to their elevated growth costs. Giant male Supermice consumed slightly less quantities of food with similar assimilation efficiencies compared to their normal siblings. Accumulated data revealed no evidence of compensatory mass-specific feeding in Supermice during either early or late growth periods. These results suggested that transgenic and normal mice operate on an equivalent base of assimilated resources despite their large differences in final adult body mass. Consequently, Supermice exist in a perpetual state of energy crisis, self-imposed by their life-long elevated levels of growth.

Many of the physiological impacts expressed by transgenic GH strains may be attributable to their intrinsic failure to adjust food intake in the face of amplified growth expenditures. Without increased resource intake, genetically engineered growth enhancement negatively impacted on other integrated elements, including low fecundity, decreased behavioural activity, and reduced longevity assurance, in accordance with the principle of allocation.

During early growth, Supermice grew at rates which did not exceed the highest recorded for normal mice (i.e. during the most rapid phase of growth, Supermice and normal

animals grew similarly). As the present results suggest, saturated levels of growth hormone did not increase growth rates beyond existing normal levels, but rather offset normal age-specific declines. Unlike their normal counterparts, transgenic mice maintained elevated rates of growth into later ages. The fact that Supermice attained greater biomass than their normal relatives on slightly smaller amounts of food suggested that transgenic animals were more efficient at food utilization. Our second crucial finding was that increased growth in Supermice was achieved via improved production efficiency as obtained by diverting resources from other metabolic demands. Of note was our discovery that these changes in efficiency were evident even before the animals diverged much in growth rate.

Although the positive relationship between measures of production efficiency (i.e. *GPE* and *NPE*) and growth rate has been widely reported, a general empirical relationship has not been previously documented. Our results confirm the strong positive correlation between rates of growth and production efficiency. An ontogenic overview revealed that production efficiency was in fact the key dimension used by mice to adjust for the costs of varying growth rates. Ontogenic alterations in physiological resource allocation were also apparent in giant Supermice during early and late growth intervals. These observations (Kajiura and Rollo 1995), not only supported our prediction of greater production efficiencies for younger more rapidly growing mice, but more importantly, clarified differences expressed by both strains at early ages. Supermice and normal mice were clearly differentiated even at younger ages (Fig. 3.8), implying that Supermice differed qualitatively from normals even when rates of growth and body sizes were similar.

The single lines resolved for late growth *GPE*'s and *NPE*'s (mice aged 47-62 days old)

strongly correlated with growth rates, which is congruent with our previous observations for mice aged 50-61 days old. It may be that transgenic GH animals represent extensions of altered resource allocation to a scope that is beyond normal *Mus musculus* evolutionary experience. Mass budget data for older Supermice and normal mice represent an independent verification of the stability of our previous findings over several generations. For the Supermouse, altered resource allocation is established prior to any divergence in body size with production efficiency playing a vital role in growth modulation. Improved efficiency of converting food into an almost twofold increase in body mass is achieved at the expense of other physiological functions. Given the negative ramifications of reduced longevity, diminished fecundity, and low behavioural activity, the so-called "improvement" in production efficiency was attained only at a tremendous burden or "stress" to the transgenic organism.

Strong similarities are evident in transgenic correlations exhibited in Supermice, genetic correlations yielded by mutations and artificial selection, and phenotypic correlations arising from environmental variation. These findings have important implications with respect to the physiological basis of the "transgenic correlation structure". The Supermouse appears to represent an extension of adaptive integration reflected by the reaction norm and genetic correlation structure of normal mice into a non-adaptive realm. Mice appear to have evolved a strategy with production efficiency and mature body sizes as the key factors adjusted to variations in growth rate while mass-specific consumption rates and metabolic rates remain relatively fixed. Obvious trends resolved from correlation and regression analyses point to the existence of a fundamental resource allocation modulator. Clearly, empirical support from the Supermouse model and literature pertaining to dietary restriction, artificial selection, mutants,

and racial variants support the contention that production efficiency is a major fulcrum in the phenotypic plasticity and evolution of *Mus musculus*.

Transgenic technology offers an immediate, goal-oriented means to emphasize or repress attributes via a specific known and usually single genetic change. Ligation of various promoters to different gene coding sequences results in transgenic organisms with genetic traits and tissue-specific patterns of gene expression, which rarely result through natural selection or via artificial breeding regimens due to genome stability (transposable elements being the exception) or interspecific biological barriers. Although the probability of generating a mouse expressing extra growth hormone genes with a unique promoter by methods other than transgenesis is remote, theoretically it is quite probable that an animal could arise carrying extra copies of growth hormone genes via transposable elements or DNA copying errors (see Rollo (1994) for review). Not only have most major gene families evolved in this way, but there are five variants in the GH gene family itself, and other genes such as prolactin are also related to GH. The diversification of these genes and unique aspects of their transcription control undoubtedly arose via gene duplications and subsequent divergence (Strobl and Thomas 1994).

Based on the lower fitness imparted by the extra GH genes of Supermice, genic theorists contend that normal mice would ultimately displace transgenics if both strains were forced to compete in nature. In contrast, proponents of coevolution maintain a more holistic perspective and caution against considering only isolated elements. Rollo (1994) suggested that any selection differential between normals and transgenics may more aptly be considered as a competition between two gene complexes varying in their coadaptation levels. Selection

may alter other aspects to support upregulated or duplicated GH genes, if large body size bestows an advantage to the animal. Selection in this case might act on correlated responses to GH, rather than simply on removal of the GH genes. That is, the unit of selection is the coadapted complex within which the growth hormone genes operate. Of great relevance here was our recent demonstration (unpublished data) that Supermice, allowed to self-select extra carbohydrate, are able to partially restore many of their compromised functions including behaviour, reproduction, and longevity. As alluded to earlier, changes in GH are intimately linked to not one but several integrated fitness features. Hence, the third insight revealed by the present investigation suggests that a more probable consequence of germ-line modifications for enhanced growth entails high-rate animals reflecting disrupted coadaptation. Restoring functional coadaptation while maintaining growth will clearly be an unavoidable part of genetic engineering.

Theoretically, Supermice could avoid extinction in the wild if they shifted their feeding niche to reduce competition with normal *Mus musculus*. The self-selection of different food to correct their metabolic imbalances could actually represent at least a partial shift. If transgenic strains were selected or genetically altered for restored reproduction and improved survival whilst preserving higher growth without compromising other physiological functions, then perhaps genomic reorganization (the initial stages of speciation) could occur. Increasing the number of genes coding for GH in *Mus musculus* is definitely not equivalent to transforming mice into rats (i.e. <u>not</u> a speciation event) but instead produces an extremely high-rate animal with disrupted coadaptation. In fact, a better appellation for the Supermouse might be the acromegalic mouse.

Went and Stranzinger (1991) insisted that if the use of transgenic breeds is envisaged in pharmaceutical, food, and fiber production, research on large domestic animals must continue and should not be substituted for by investigations employing the "much cheaper mouse model". However, our results support the utility of the Supermouse model in providing valuable and practical insights into the effects of transgene induction. If engineered "improvements" in large domestic livestock are desired, extensive examination of the effects of specific transgenes in smaller laboratory animals should be encouraged to first identify potential problems.

Comparisons of our results with that of other transgenic GH strains, artificially selected mice, and various experimentally manipulated animals injected with exogenous GH reveal several fundamental similarities but also some key differences. Possible explanations of the varying impacts include differences in (*i*) the strain of mice used, (*ii*) the expression of the incorporated promoters and gene constructs, or (*iii*) the biological activity of GH's derived from various species. Extrapolation of our Supermouse results to larger species also requires careful consideration of species' differences in resource intake and body composition.

Since GH may act indirectly or directly on multiple targets at reproductive, hypothalamic, gonadal, and pituitary levels, functional aberrations exhibited by transgenic growth-enhanced organisms may represent not only primary effects of GH, but also indirect actions of GH (e.g. impacts on neurotransmitters, insulin resistance, plasma glucose, fatty acid levels, IGF-I and IGF-II). Thus, further research is warranted to discover methods to modulate the time and tissue-specific expression of incorporated transgenes (e.g. other promoters, hormones, or external stimuli). Although many facets of how various life-history

components interface have yet to be completely elucidated, the present resource allocation studies offer several practical insights into aspects relevant to the "transgenic correlation structure".

Within the past decade, transgenic technology has successfully transcended *in vitro* cell-cultured studies into the domain of *in vivo* whole-organism systems revealing exciting discoveries in several specialized areas of research. Supermice, and transgenic animals in general, provide powerful new tools for testing life-history theory, which has previously been restricted to phenotypic correlations in normal animals, or genetic correlations in artificial breeding strategies. The Supermouse represents a new and highly precise probe for detecting trade-offs, and clearly illustrates the operation of the principle of allocation. The evidence described here underscores the value of applying holistic ecological vision to understand and perhaps further improve genetically engineered organisms. The power of the Supermouse model and the scope of questions we may explore employing it rests upon how well it is characterized. Only through consorted efforts merging diverse scientific fields will we successfully understand genetically engineered organisms to fully reap the vast rewards that lie waiting.

SECTION V

GENERAL LITERATURE CITED

5

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 fiber 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, pyridoxine hydrochloride, ferrous sulfate, vitamin supplement, vitamin D-3 supplement, vitamin E supplement, calcium iodate, ferrous carbonate, copper sulfate, zinc oxide.

APPENDIX A (cont.)

STANDARD LABORATORY RODENT DIET™

LABDIET® 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

APPENDIX A (cont.)

Neutral Detergent Fiber %	16.0
(cellulose, hemicellulose, lignin)	
Acid Detergent Fiber %	8.2
Total Digestible Nutrient %	76.0
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

APPENDIX A (cont.)

Iodine, ppm	0.7
Chromium, ppm	1.83
Selenium, ppm	0.20
Vitamins	
Carotene, ppm	4.5
Menadione (added), ppm	--
Thiamin, ppm	15.0
Riboflavin, ppm	8.0
Niacin, ppm	95.0
Panthothenic Acid, ppm	24.0
Choline, ppm × 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

General Rearing Container:

B.1 Angled View (cage assembled)

B.2 Top View (cage disassembled)

t = stainless steel grill hopper

w = glass water bottle

c = clear plastic container (l x w x h = 27 x 21 x 15.5 cm)

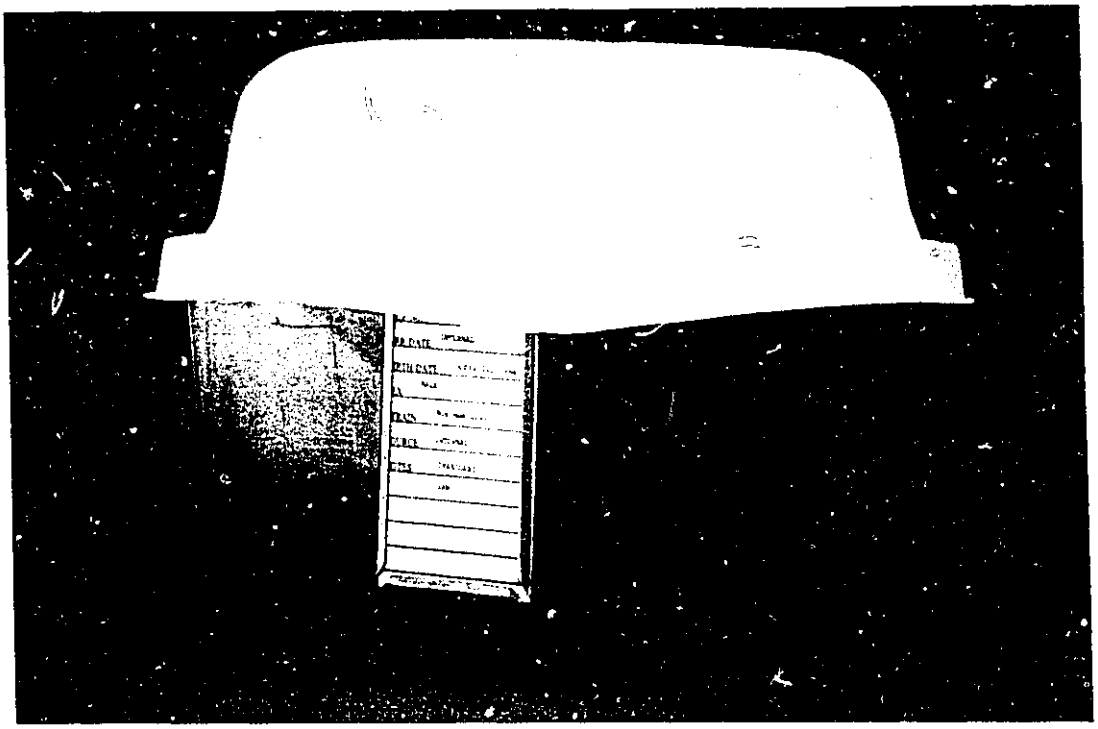
i = identification card and card holder

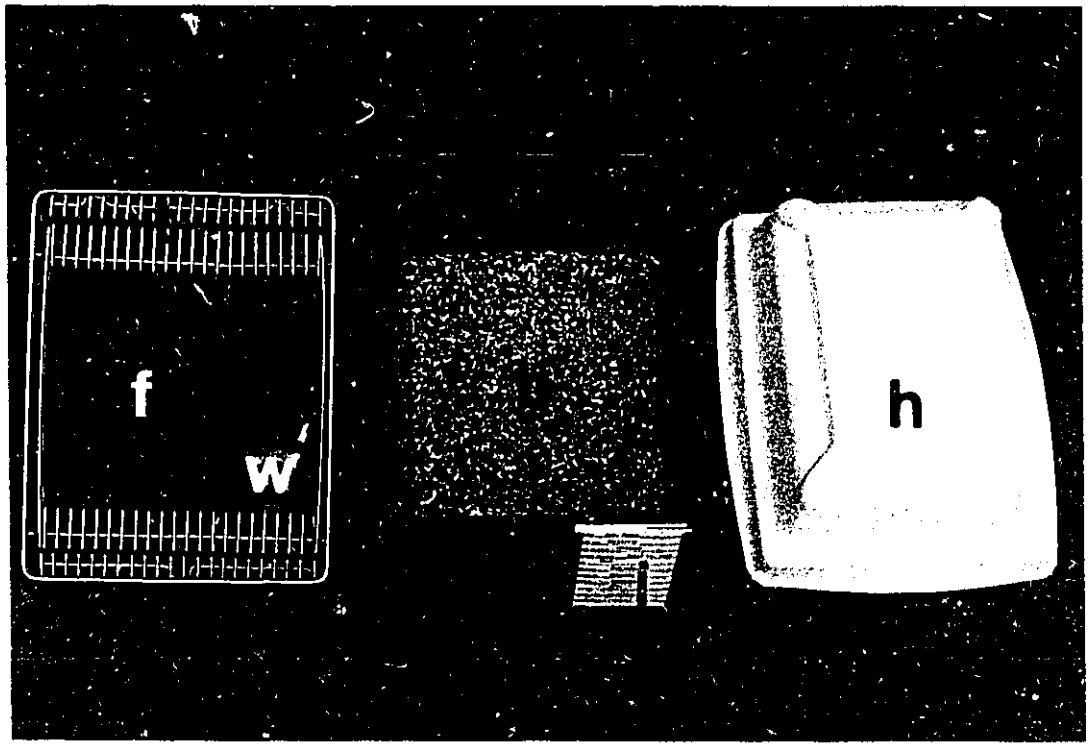
h = air filter hood

f = food pellets (Lab Diet® No. 5001, PMI Feed Inc.)

b = bedding material (Beta-Chip®, Northeastern Products Corp.)

APPENDIX B (cont.)





APPENDIX B (cont.)

Experimental Mass Budget Container:

B.3 Angled View (cage assembled)

B.4 Top View (cage disassembled)

t = stainless steel grill hopper

w = glass water bottle

c = clear plastic container (l x w x h = 27 x 21 x 15.5 cm)

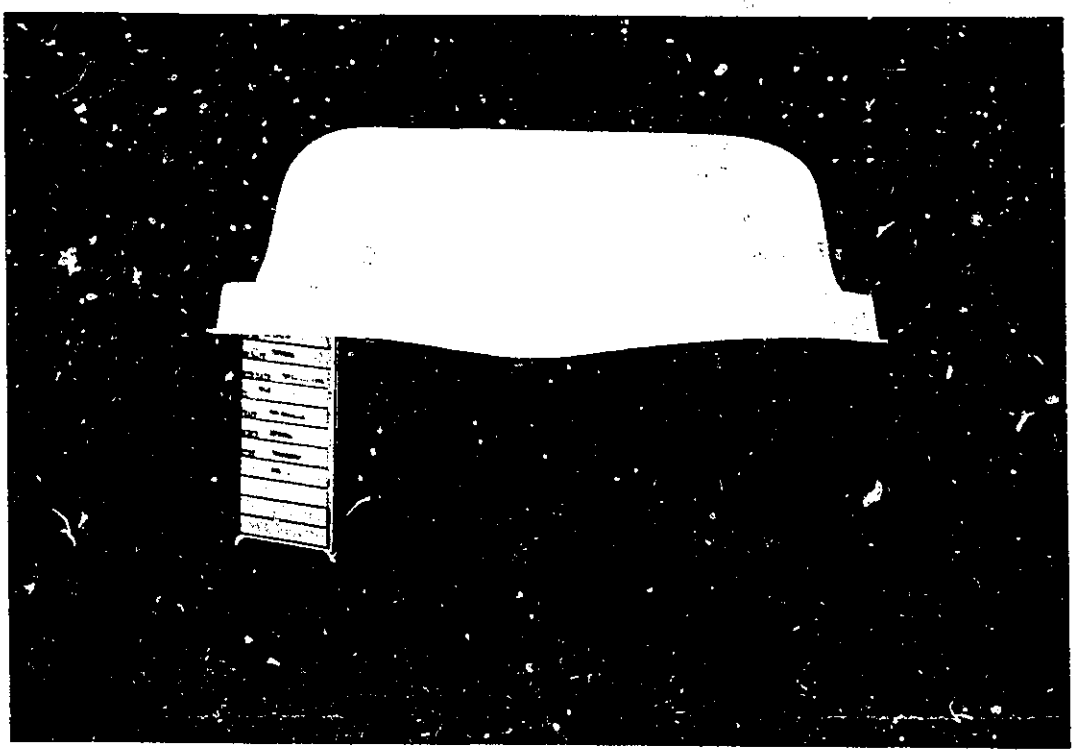
i = identification card and card holder

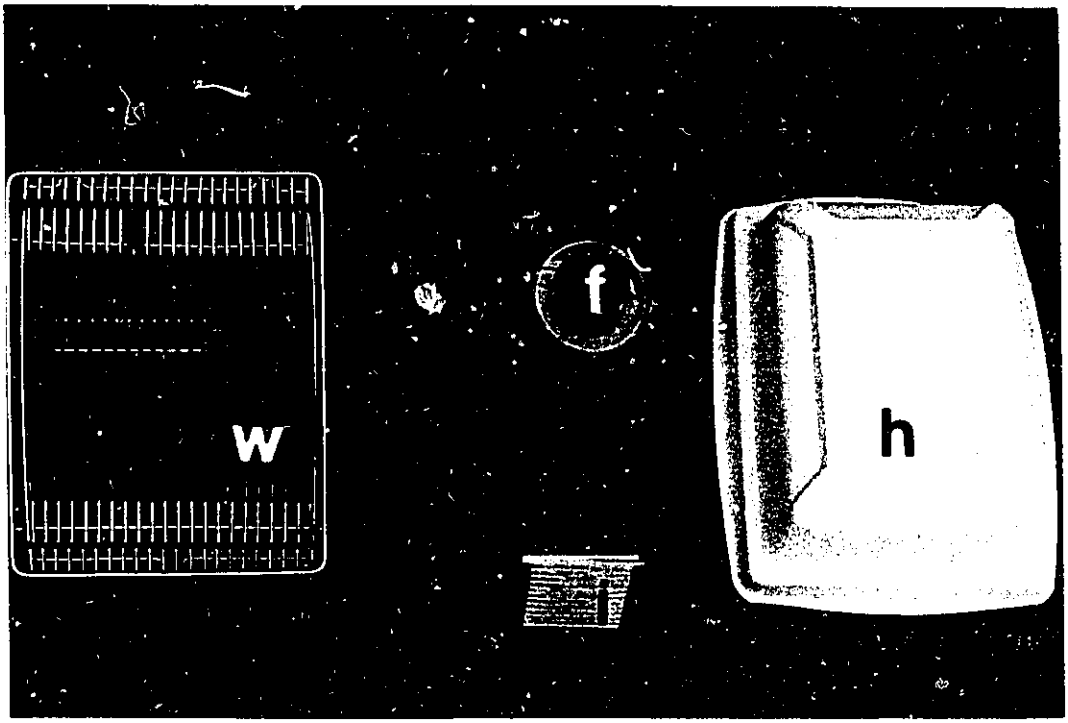
h = air filter hood

f = food pellet dish (Lab Diet® No. 5001, PMI Feed Inc.)
Pellets are secured to a 5.3 cm plastic petri dish with silicone adhesive. Pellet dish is placed on a 9 cm plastic petri dish and bound with 18 gauge copper wire.

p = steel grid platform

APPENDIX B (cont.)





APPENDIX B (cont.)

B.5 Male transgenic Supermouse in mass budget container.

APPENDIX B (cont.)